VOLUME 56 [J. CELL. AND COMP. PHYSIOL.] NUMBER 3

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

DECEMBER 1960

PUBLISHED BIMONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
THIRTY-SIXTH STREET AT SPRUCE, PHILADELPHIA 4, PA.

Second class postage paid at Philadelphia, Pa.

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mperature Studies Implicating Calcium in gulation of Muscle Membrane Potential[1]

JULIA T. APTER AND K. KOKETSU
Neuropsychiatric Institute of Illinois, Chicago, Illinois

According to the Nernst equation, the ential across a semi-permeable mem- ne separating solutions of dissimilar nposition is a positive linear function temperature. Attempts to demonstrate t this equation also describes the be- vior of the intact cell membrane appar- ly succeeded for muscle (Ling and odbury, '49; Jenerick and Gerard, '53) failed for nerve axon (Hodgkin and z, '49). On the other hand, Goldman 3) has indicated, on theoretical grounds, t temperature might differentially alter permeability of the cell membrane to assium, sodium, and chlorine ions, mak- potential a complex function of tem- rature. Neither the Nernst nor the Gold- n equations, however, are compatible h several types of recent evidence. eat reduction (Tobias, '50) or increase rundfest et al., '54; Folk and Gerard,) in internal cellular potassium did not er membrane potential to the extent dicted by these equations. For similar sons, Shaw et al. ('56), Stephenson 7), and Koketsu and Kimura ('60), have ected the ionic explanation for the ori- of the membrane potential. What is re, the data of Stampfli ('59) and of Ling 0) also conflict with the ionic hypoth- s. The temperature-potential relation- p to be reported here adds to the body evidence refuting the applicability of equations to the cell membrane po- tial. The present experiments indicate. ead, that the concentration of calcium r at the cell surface controls membrane ential. Other ions and temperature alter ential by changing the concentration of mbrane calcium and not by a Nernst or dman effect.

METHOD

The sartorius muscles of winter frogs limated to 5°C were removed with care, especially to avoid crosscutting fibers. After immersion in frog Ringer's solution for one hour at 5°C, specimens were continually irrigated for two to 6 hours with a test solution also at 5°C. The composition of solutions are listed in table 1; all except sucrose were buffered with tris brought to pH 6.8 with HCl. Visualized cells on the inner exposed surface of the muscle were impaled with micropipettes (0.5 μ diameter) filled with 3 M KCl. A variety of of temperatures could be reached quickly and maintained indefinitely by irrigating from two reservoirs, one at 3° and the other at 30°C. By impaling only surface cells and by continual irrigation, the response of the resting membrane potential to temperature in the presence of a known external ionic concentration could be measured. Over a thousand measurements were made on 84 muscles. Responses of potential to a change in temperature were noted during impalement of a single cell and also by statistical survey of measurements on 10 or more cells.

Several muscles were tested without irrigation in 5 mM KCl Ringer in order to repeat the experiments of Ling and Woodbury ('49).

RESULTS

These will be divided to treat separately (A) the time course of the response to an abrupt change in temperature, and (B) the steady state temperature-potential relationship.

A. Time course

The resting muscle membrane potential responded to abrupt changes in temperature with a bi- or triphasic transient which attained a steady state in about 20 minutes. A rise in temperature from any level

[1] This work was supported by U.S.P.H.S. grants B-2708 and B-1650 and by a grant from the Illinois Department of Public Welfare.

TABLE 1

Name	Concentration in mM/liter				
	Na^+	K^+	Ca^{++}	Cl^-	Sucrose
Ringer	112	2	1.8	117.6	
5 mM K-Ringer	112	5	1.8	120.6	
Ca-free Ringer	112	2		114	
Low Ca Ringer	112	2	0.18	114.36	
K-free Ringer	112		1.8	115.6	
Isotonic sucrose					224
K-free sucrose			1.8	1.8	224
Ca-free sucrose Ringer		2		2	224
Na-free Ringer (sucrose Ringer)		2	1.8	5.6	224
Saline	112			112	
30 mM K-Ringer	82	30	1.8	115.6	

TABLE 2

Solution	Temp.	Potential		Reversible
		Mean	s.d.	
	°C	*mv*	*mv*	
Low Ca Ringer	5	92	3	+
	26	81	4	
Ca-free Ringer	5	93	3	−
	26	52	6	
Ca-free sucrose Ringer	5	37	2	
	26	38	2	
K-free Ringer	5	116	4	
	26	114	4	
Na-free Ringer (sucrose Ringer)	5	45	3	
	26	47	2	
K-free sucrose	5	112	5	+
	26	96	4	
Saline	5	120	7	±
	26	102	7	
Isotonic sucrose	5	138	10	+
	10	131	10	
	15	119	8	
	26	100	7	
Ringer	5	111	2	
	15	100	2	
	26	109	2	
5 mM K-Ringer	10	72	4	
	25	73	3	
30 mM K-Ringer	5	38	2	+
	26	32	3	

below 30° induced, initially, a rise in potential, vice versa a drop in temperature, if calcium were present or in calcium-free solutions also free of potassium ions (saline). In contrast, in calcium-free solutions containing potassium, potential dropped precipitously when temperature rose. If sodium were also present in these latter solutions the muscles twitched constantly at 26° but not at 5°.

B. Steady state temperature-potenti relationships

These results are summarized in tab showing that even after 6 hours irriga with any of the test solutions (excep

M KCl) at 5°C, potentials were at or ove the 90 mv expected for frog sartoius muscle. In not one instance was the tential higher at 26°. Rather, in solutions containing calcium, potential appeared to be independent of temperature in e range tested, although transient anges in potential did occur with anges in temperature. This lack of dendence, in the steady state, between mperature and potential held for Ringer K^+ concentrations 0, 2, and 5 mM) and r sucrose solutions containing K^+ with or ithout Ca^{++}.

In solutions containing little or no calum or in 30 mM KCl, which appeared to duce the effective calcium concentration, tentials were lower at 26° than at 5°. he drop in potential brought on by warmg was most striking in Ca^{++}-free Ringer containing K^+ and Na^+). In this solution uscles started to twitch and potential opped precipitously when temperature se (gradually or abruptly) from 5° to 6°. In K^+-free solution which was also a^{++}-free (saline) the potential was very gh at 5° and only slightly lower at 26°, spite of spontaneous twitching. In soum-free solutions that were also K^+-ee (sucrose with Ca^{++}), there was no vitching at 26° but potentials were lower an at 5°. In pure sucrose, where potasum and sodium as well as Ca^{++} were ithheld, the negative temperature-potenal relationship was also observed.

A negative relationship existed, therere, between temperature and the potenal across the cell membrane in environents containing less than 1.8 mM Ca^{++} in 30 mM K^+. This relationship was not rictly reversible if Ca^{++} were completely sent and either Na^+ or K^+ were present. was, however, strictly reversible in all lutions containing some Ca^{++}, however nall, and in Ca^{++}-free solutions which ere also free of K^+ and Na^+, being only metimes reversible if Na^+ alone were esent.

DISCUSSION

Altering the temperature of Ringer's lution did not appreciably change memane potential, in apparent disagreement th Ling and Woodbury ('49) and Jenerk and Gerard ('53). In some of the esent experiments however, especially when irrigation was not satisfactory, a slight positive temperature-potential relationship could be found, but this was not reversible. Since it is generally agreed that measurements of membrane potential are most reliable when the cell surface is continually irrigated by a test solution, the results of the present experiments call for a reconsideration of earlier measurements of the effect of temperature on potential and they suggest that the Nernst and Goldman hypotheses are not applicable to the living cell membrane. Rather, when the present experiments are viewed in the light of other work from this laboratory, the mechanism governing membrane potential seems to involve an interaction of calcium, incorporated in the membrane, with external calcium and potassium.

The other work has shown, for example, that radiocalcium loaded on muscles leaks out at room temperature, probably being replaced by external potassium when calcium is not available from the medium or when external potassium concentration is high (Koketsu and Miyamoto, '60). Work now in progress indicates, moreover, that the membrane potential is a function of the net loss of calcium from the cell (probably the cell surface). It is reasonable to assume from these results that replacement of calcium by external potassium can bring about a drop in potential.

The findings of the present study coupled with these other data suggest that calcium may be held in the membrane more or less firmly, depending on temperature; that the more weakly-bound form (Ca_m) can be replaced by external potassium. Potential seems to be the greatest when the surface concentration of the more firmly-bound calcium (Ca_M) is highest. The relation between these forms of membrane calcium and external Ca^{++} and K^+ concentrations may be explained by the following scheme:

$$Ca_M \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Ca_m \qquad (1)$$

$$Ca_m + 2K \underset{k_4}{\overset{k_3}{\rightleftharpoons}} 2K_m + Ca \qquad (2)$$

where K_m indicates that potassium has replaced the less firmly bound calcium in the membrane, and k_1, k_2, etc. refer to the rate constants of the various reactions. It

is assumed that all rates increase with temperature; the temperature coefficient of k_1 and k_4 being greater than of k_2 and k_3 respectively. Admittedly there is no direct evidence to support these particular assumptions, but they are consistent with the present findings and are not inconsistent with any information now known about the behavior of the membrane.

Equation 2 indicates that the extent to which potassium enters the membrane would be a function of temperature and of a balance between the concentrations of environmental calcium and potassium, as well as of the density of Ca_m in the membrane. At low temperature, the low ratio of k_1 to k_2 would make the concentration of Ca_m low, shifting reactions 1 and 2 to the left, keeping potential high. External potassium ion concentrations would regulate reaction 2; the more K^+, the greater the shift to the right, using up Ca_m supplied by reaction 1. The associated reduction in Ca_M would reduce membrane potential commensurate with the potassium concentration. A sufficiently high calcium concentration could counterbalance this effect by shifting reaction 2 to the left. In solutions free of these ions or with Ca^{++} alone, only equation 1 need be considered. There would be no loss of Ca_m, so that Ca_M, the governor of potential, would be at maximal concentration and a high membrane potential could be found.

At 26°C, all rates would be faster, the ratio of k_1/k_2 being higher than at 5°, making the concentration of Ca_M lower, Ca_m higher and shifting reaction 2 to the right. However, the ratio k_3/k_4 is assumed to be lower at 26° than at 5°, although individual rates are faster, tending to shift reaction 2 to the left. The net result could be that K_m and Ca_m would not be changed with temperature, so long as calcium and ordinary amounts of potassium were present. In contrast, a calcium-free or potassium-rich environment could shift reaction 2 sufficiently far to the right to counterbalance the thermal effect of the rate constant ratio. In Ca^{++}-free solutions for example, at 5° the potential could depend on the amount of K^+ available to replace membrane Ca^{++}, high potentials occuring at low concentration of K^+. At 26° the replacement of K^+ would be greater with no Ca^{++} available to maintain equilibrium in reaction 2 and an reversible drop in potential would res The observed precipitous drop in poten when temperature was raised is consist with this conjecture. In potassium-r solution containing Ca^{++}, potential, pendent on the potassium concentratti would be low at 5° but even lower at where more K_m would be present at equ brium, pulling both reactions 1 and 2 to right, reversibly. In ion-free solutions K^+ is available to enter the membrane only reaction 1 need be considered. these two latter situations, more Ca_m wo be present at 26° than at 5°, giving the versible negative temperature-potential lationships found here.

The presented hypothesis is, therefo consistent with the observations on temperature-potential relationship and with known facts about the effects of ternal potassium ion concentration membrane potential. Indeed, there are facts available at the present time wh suggest that the hypothesis is untenabl

SUMMARY

1. Measurements of the membrane tential of unstimulated frog sartorius m cles were made at several temperatures tween 5° and 26°C during contin irrigation by solutions containing vari concentrations of Ca^{++}, K^+, Na^+ and

2. The transient and steady state sponses of potential to a change in t perature were noted during impalemen a single cell and also by statistical sur of measurements on 10 or more cells.

3. In solutions containing 1.8 mM cium and the usual amount of potassi the steady state potential was independ of temperature.

4. In solutions with little or no calci a negative relationship existed betw temperature and potential which strictly reversible in Ca^{++}-free solution Na^+ and K^+ were also absent, in Ca poor solutions, and in K^+-rich soluti containing 1.8 mM Ca^{++}. The nega relationship held, but was only someti reversible if Na^+, but not K^+ was pre in Ca^{++}-free solutions.

5. These results permit a re-evalua of the applicability of the Nernst and G man equations to the cell membrane, gesting, instead, that the concentratio

cium in or at the cell surface controls mbrane potential.

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n the ATPase Activities in the Retina and e Rod Outer Segments[1]

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We have very little information as to the Pase activity of the retina in connection th the visual process, though some attenn has been paid to the existence of this zyme in the retina (De Berardinis and riccho, '51; Hayano, '52; Majima, '53). e present investigation was intended to cidate the effect of illumination on the Pase activity of the whole retina and at same time to examine the properties of s enzyme activity in detail. The investiion was further extended to the ATPase ivity of the rod outer segments likewise der the influence of light, because this rtion was known as containing rhodop- and accordingly might be regarded as e of the most essential parts of the ret- in regard to the visual process.

MATERIALS AND METHODS

Preparation of the retinal homogenate and the rod outer segments

In all the experiments, cattle retinas re used. The excised cattle eyes kept in ice-cold container were brought from slaughter-house to the laboratory and retinas were isolated under a dim red np in the dark room.

For preparation of the homogenate, 10 inas were put into 10 ml of ice-cold % NaCl and homogenized for three nutes at 25,000 rpm with the stainless el homogenizer. Appropriate aliquots of s homogenate were used for the deternation of the ATPase activity.

The separation of rod outer segments o.s.) was performed by the modified hnique of Collins et al. ('52). The terials were derived from the sugar er with specific gravity between 1.12 d 1.13. After two times washing with % NaCl, the r.o.s. were suspended in ine, appropriate aliquots of which were used for the determination of the enzyme activity. From the microscopic observation, the other fragments than r.o.s. could be estimated as much as about 1% so that the impurities would be negligible for participating in the enzyme activity.

Determination of the ATPase activity

The sodium salt of ATP was supplied by courtesy of Okunuki's Laboratory (Imamoto et al., '58). It contained less than 2% inorganic phosphate. Ba-salt used in the early experiments was washed with ice-cold acetate buffer (pH 4.0), dissolved in a small volume of 0.1 M HCl. After precipitation of Ba with Na_2SO_4, the solution was neutralized with dilute NaOH.

Histidine buffer (final conc. M/60) was used in most of the present experiments, in the early experiments veronal and glycylglycine buffer (both final conc. M/50). Every buffer solution gave almost the same optimum pH.

All the determinations of the enzyme activity were done in duplicate; 0.5 ml of the homogenate or the suspension was added to 0.5 ml of the buffer solution. After 10 minutes preincubation, the reaction was started by addition of 0.3 or 0.5 ml ATP, if desired, 0.1 ml $MgSO_4$ (final conc. 7.7 mM) and 0.1 ml KCl (final conc. 7.7 mM), and further 0.154 M NaCl to make up a total volume of 2 ml. After incubation at 37.5°C, 2 ml of ice-cold 10% trichloroacetic acid was added to stop the reaction. The mixture was then centrifuged for 10 minutes at 4,000 rpm and inorganic phosphate liberated from the supernatant was measured by the modified method of Fiske and Subbarow ('25).

[1] This investigation was supported in part by a Grant-in-Aid for the Miscellaneous Scientific Research from the Ministry of Education.

In order to examine the effect of illumination, the isolated retina was divided into two equal parts through the section including the optic nerve papilla, one used as the illuminated sample and the other as the control (dark). The materials above mentioned were put into a beaker containing 30 ml of 0.9% NaCl to be illuminated with 3,000 Lux for 15 minutes. After illumination, the materials were stored in ice-cold 0.9% NaCl and used for the preparation of homogenate and the isolation of r.o.s.

RESULTS

A. The ATPase activity of the retinal homogenate

From the preliminary experiments the following results were obtained.

1. The rate of reaction is proportional to the concentration of homogenate so far as 1–4 mg dry weight/ml or 0.1–0.4 mg in total nitrogen content.

2. The enzyme activity is linear with time for initial 30 minutes and then decreases gradually.

3. The enzyme activity is optimum at the concentration of substrate (ATP) more than 3 mM.

4. The optimum pH is 6.8 at the range between pH 6.2 and 8.6. In the rabbit retina the activity of ATPase was proved as optimum at pH 9.1 (De Berardinis and Auriccho, '51). It is not clear if another optimum pH exists in the range over 8.6. Anyhow, the optimum pH is not varied the use of different buffer.

5. The enzyme activity is inhibited PCMB (10^{-5} M) and Ca-ion (10^{-3} M), affected by KCN (1.5×10^{-2} M) and (5×10^{-4} M). DNP (5×10^{-4} M) has effect on the activity in the presence Mg-ion.

Effect of divalent cations. Table shows the results obtained from the experiments on the influence of Mg-ion and ion. Mg-ion accelerates the enzyme activity by more than 100%, while Ca-ion scarcely effective. It seems that the activity of Mg-activated ATPase is especially intensified in the retinal homogenate. the presence of both Mg- and Ca-ion equal concentration, the enzyme activity falls so remarkably that this enzyme seems to be similar to that of the microsomal fraction observed by Kielley and Meyerhof ('48).

Effect of K-ion. The ATPase activity the retinal homogenate was measured the presence of Mg-ion and K-ion instead Na-ion. The activity is considerably ered in the range over 77 mM of K-ion 11% at 77 mM and 28.3% at 115 (table 2).

ATPase activities under various K-level. It is of interest to examine whether ATPase activity changes correspond the changes in the K-level of the retina tissue, since the activity is found as be

TABLE 1

Effect of Mg- and Ca-ion on ATPase activity in retina and rod outer segments

Concentration of ions		ATPase activity			
		Retina			R.O.S.
Mg-ion	Ca-ion	No. 1	No. 2	No. 3	
mM	*mM*	*μg P/mg dry weight*			*μg P/mg N*
0	0	43.5	31.6		16.0
0.77	0			25.8	34.2
1.54	0	91.6	68.4	28.5	37.8
3.85	0		63.7	28.0	37.0
7.7	0			23.2	
15.4	0			20.5	27.2
0	0.55	43.5			
0	1.54		37.0		13.2
0	2.75	47.6			
0	5.5	40.3			14.0
1.54	1.54	31.0			23.6

Reaction medium: ATP, 3.3 μmol for retina and 1.8 μmol for r.o.s.; K-ion, 7.7 mM; glycylglycine buffer, M/50 (pH 6.8). Incubation period; 15 minutes for retina and 5 minutes for r.o.s.

TABLE 2

Effect of K-ion on ATPase activity in retina and rod outer segments

K-concentration	ATPase activity (μg P/mg N)		
	Retina	R.O.S.	
		No. 1	No. 2
mM			
0	74.0	29.6	
0.77	75.2		
1.54		37.7	
3.85	76.4	39.6	
7.7	74.8	45.8	59.4
38.5	71.0	49.5	
77	68.0	43.6	
101			44.8
115	54.8	34.0	
154			14.2

Reaction medium: ATP, 3.3 μmol for retina and 1.8 μmol for r.o.s.; Mg-ion, 1.54 mM; histidine buffer, M/60 (pH 6.8). Incubation period: 10 minutes for retina and 5 minutes for r.o.s.

ɔendent on the K-concentration of the ction medium. As noticed in the previ- report (Sekoguti, '60a), the K-level of tle retina becomes higher through the ubation with glucose and glutamate, high in the medium containing glu- e only. Thus the ATPase activities of retinal homogenate were determined ler various K-levels prepared by pre- atment of the tissue with glucose and tamate. The results are summarized in le 3. Since the experimental error is imated less than ± 3%, the differences sented in table 3 can be regarded as nificant. Thus the higher the K-level of ue, the lower the enzyme activity be- nes, though not considerably. As shown table 3, the enzyme activity in exp. 2d in spite of the higher K-level, higher than in exp. 2c. It can be supposed that there exists probably the optimum K-level as well as the optimum K-concentration of 3.85 mM for the enzyme activity (table 2).

B. The ATPase activity of the rod outer segments

The homogenate was prepared from the other tissue fragments than r.o.s. and its ATPase activity was compared with that of r.o.s. The result shows that the latter is on the average 1.27 times higher than the former. As for the ATPase activity of r.o.s. suspension, we found the following facts.

1. *Concentration of enzyme.* So far as the nitrogen content of r.o.s. suspension is 0.3–0.9 mg/ml, the rate of reaction is proportional to the enzyme concentration (fig. 1).

TABLE 3

ATPase activity of retina under different K-levels

Exp. no.	K-content	ATPase activity	Difference
	meq/gm N	*μg P/mg N*	*%*
1 a.	4.14	79.8	
c.	2.52	86.7	+ 8.6
2 a.	4.03	97.0	
b.	2.73	103	+ 6.2
c.	1.66	105	+ 8.2
d.	1.95	108	+11.3

The retina was cut to two and 4 equal portions in exp. 1 and 2 respectively. Each portion was incubated for 40 minutes at 37.5°C in Krebs-Ringer bicarbonate (a) with 0.2% glucose and 10^{-2} M Na-glutamate, (b) with 0.2% glucose and (c) with the same medium as (a) but without K-ions. After incubation except in exp. 2 (d) without incubation, the ATPase activity was determined.

2. *Time of incubation.* In the relatively lower concentration of enzyme (0.174 mgN), all the terminal phosphates of ATP are liberated during the initial 10 minutes of incubation and then the activity decreases sharply (fig. 2). This low activity may be probably due to the activity of adenylate kinase existing together.[2] It may be reasonable to consider that the linear portion of the activity curve could be ascribed to the activity of ATPase itself. Thus we adopted 5 minutes incubation for further experiments.

3. *Concentration of substrate.* The enzyme activity is proved as being optimum above 1.75 μmol of the substrate concentration (fig. 3).

4. *Optimum pH.* In glycylglycine buffer solution, the enzyme activity is maximum at pH 6.8 (fig. 4). Histidine buffer gives also the same optimum pH.

5. *Effect of Mg- and Ca-ion.* Under the present experimental conditions, the enzyme activity is accelerated by Mg-ion and rather inhibited by Ca-ion (table 1). In addition, Mg- and Ca-ion have the competitive effect with each other in the equal concentration so that one must recog some difference among the effects of ion on the ATPase activity in the r.o.s. in the retinal homogenate.

6. *Effect of inhibitor.* The Mg-a erated activity is inhibited by NaF (M), IAA (10^{-3} M), PCMB (10^{-4} M) Cu-ion (10^{-3} M), but not by KCN (10^{-}

7. *Effect of temperature.* The act was measured at different temperat

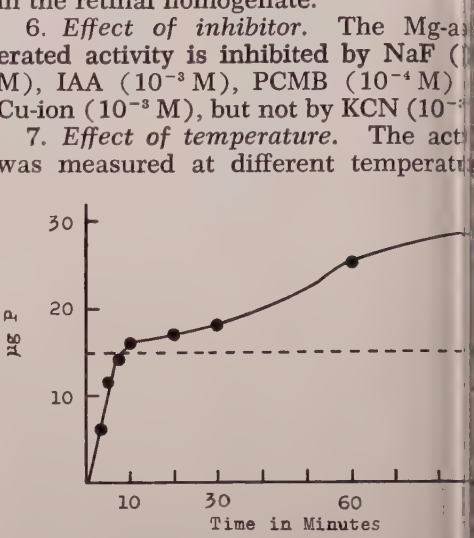


Fig. 2 Liberation of inorganic phosphate ATP. The broken line indicates the level o terminal P of ATP. Reaction medium: enz 0.174 mg-N; Mg-ion, 1.54 mM, KCl, 7.7 mM

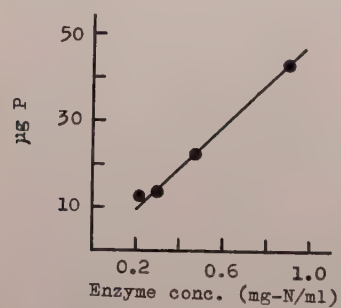


Fig. 1 Relation of enzyme concentration (nitrogen content of r.o.s. suspension) to inorganic phosphate liberated from ATP. Reaction medium: ATP, 2.5 μmol; Mg-ion, 3.85 mM; KCl, 7.7 mM.

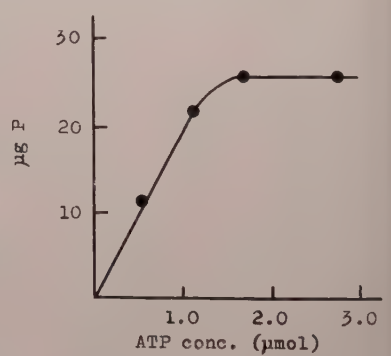
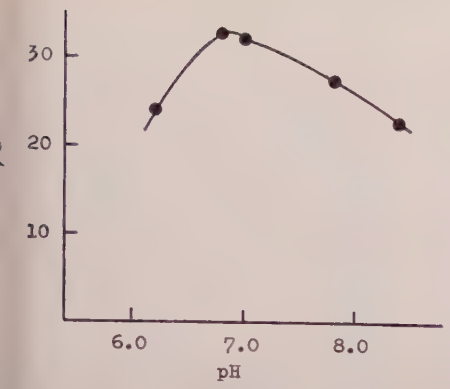


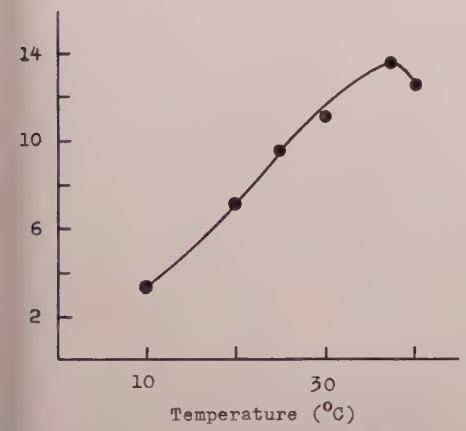
Fig. 3 Enzyme activity in relation to the centration of ATP as substrate. Reaction med Mg-ion, 3.85 mM; KCl, 7.7 mM.

[2] In the preliminary experiment, the activity of adenylate kinase in the r.o.s. was examined With the medium including ADP and glucose-hexokinase system we obtained the following results.

	Incorporated P			Phosphatase
	0.4 ml	Suspension 0.2 ml	0.1 ml	
	μg	μg	μg	μg
Normal ($MgCl_2$)	99.3	—	24.8	0
Normal ($CaCl_2$)	49.5	30.6	15.3	0
Acid treatment ($MgCl_2$)	45.2	27.9	9.5	0
Acid treatment ($CaCl_2$)	71.3	28.3	0	0



ig. 4 Relation between the enzyme activity pH. Reaction medium: ATP, 1.8 μmol; Mg- 1.54 mM; KCl, 7.7 mM; glycylglycine buffer, 50.



ig. 5 Enzyme activity in relation to tempera- of incubation. Reaction medium: ATP, 1.8 ol; Mg-ion, 3.85 mM; KCl, 7.7 mM.

e results obtained indicate that the opti- m temperature exists at 37.5°C (fig. 5).

3. *Instability of the enzyme.* Even if r.o.s. suspension is stored at 0–5°C, the yme activity has decreased already er several hours and on the next day the loss amounts to about 20% of the initial activity. After refrigeration with liquid air, the enzyme activity is found as less than 50%.

9. *Separation of ATPase from pyrophosphatase (P-Pase).* After centrifugation of the r.o.s. suspension at 4,000 rpm (2,500 g) for 20 minutes at 0°C, the activities of ATPase and P-Pase were examined for both supernatant and residue. The ATPase activity appears mainly in the residual fraction (76%), P-Pase in the supernatant fraction (72%). Thus the ATPase found in the r.o.s. can be separated from the co-existing P-Pase.

10. *Effect of K-ion.* The enzyme activity is maximum at 38.5 mM of K-concentration (table 2); it is less than 25% of the maximum value at 154 mM. The fact that the optimum K-concentration differs from that in the case of tissue homogenate may possibly be due to the difference between the preparation methods.

C. Effect of illumination on the ATPase activity

Five retinas were prepared within one hour after decapitation and the ATPase activity was determined. Table 4 shows the results. Since the experimental error is estimated as less than ± 2.0%, the enzyme activity of the illuminated retina must be considered to be slightly higher than that of the control (the dark) except exp. 1. Thus the ATPase activity tends to be accelerated by illumination of the retina.

The influence of illumination on the ATPase activity of r.o.s. was likewise investigated. As shown in table 5, the ATPase activity of r.o.s. is also considerably accelerated by illumination, far more than in

TABLE 4

Effect of illumination on ATPase activity of retina

Exp. no.	Incubation period	ATPase activity		Difference (I-D)/D, %
		Illuminated (I)	Control (D)	
	min.	*μg P/mg N*	*μg P/mg N*	
1	10	54.0	54.7	− 1.3
2	10	102.8	90.4	+13.7
3	10	116	100	+16.0
4	10	78.2	73.5	+ 6.4
	15	93.2	89.0	+ 4.7

Reaction medium: ATP, 3.3 μmol; Mg-ion, 1.54 mM; histidine buffer, M/60 (pH 6.8).

TABLE 5

Effect of illumination on ATPase activity of rod outer segments

Exp. no.	Incubation period	ATPase activity		Difference (I-D)/D, %
		Illuminated (I)	Control (D)	
	min.	*μg P/mg N*	*μg P/mg N*	
1	3.5	26.4	23.9	+10.5
	5	36.1	31.0	+16.5
2	5	33.6	28.7	+17.0
3 a.	5	37.7	36.2	+ 4.15
b.	5	13.5	12.3	+ 9.8
4	5	28.5	23.9	+19.5
5	5	11.2	10.0	+12.0
	10	24.8	22.4	+10.7
6	5	25.5	24.4	+ 4.5

Reaction medium: ATP, 2.46 μmol; Mg-ion, 1.54 mM; histidine buffer, M/60 (pH 6.8). In exp. 3b, measured after storage of two days.

the retinal tissue. The experimental error is estimated as less than ± 1.6%.

DISCUSSION

The ATPase activity found in the retinal homogenate resembles the activity of Mg-activated ATPase observed in the microsomal fraction of muscle by Kielley and Meyerhof ('48). Abood and Gerard ('54) found also the Mg-activated ATPase in the microsomal fraction of the peripheral nerves (sciatic, branchial and spinal nerves together). The ATPase activity of retina may perhaps be due to the same kind of Mg-activated ATPase.

In general the properties of ATPase have been investigated preferentially with the mitochondria of cells and with myosine A or B. Since any mitochondrial structure is not found in the r.o.s. of cattle retina from the electron microscopic observation (Sjöstrand, '53; Tanaka, '60), it must be concluded that the r.o.s. are responsible for the ATPase activity concerned in the present experiment.

This enzyme activity is considered to be closely connected with the structure of r.o.s., because it is unstable against temperature like the activity of the Mg-activated ATPase from the microsomal fraction of muscle and furthermore the enzyme is not to be easily extracted with saline. As to the effect of Mg- and Ca-ions as well as of inhibitors, we found some differences between the ATPase activity in the r.o.s. and that in the retinal homogenate. Such differences may be due to some differences in the spatial organization of enzyme proteins.

As shown in tables 4 and 5, the ATP activity in the retinal tissue and the r.o. significantly accelerated by illuminat Auriccho and De Berardinis ('51) m ured the activity of adenylpyrophospha in retina after *in vivo* illumination, could find no effect of illumination. I supposed that a slight effect of illum tion may be impossible to determine un their experimental conditions.

From my previous reports that the K-loss from retina is increased by illum tion (Sekoguti, '60b) and on the contr the K-uptake of retina decreases thro illumination (Sekoguti, '60a), it may reasonable to consider that K-ions are leased from the retina under illuminat On the other hand, table 2 indicates t the ATPase activity depends on the K-c centration in the reaction mixture; it creases at the higher K-concentrati Thus it is considered that the K-loss fr the retina is increased by the effect of li and this plays a role of trigger for incre of the ATPase activity in the retina.

However, the K-amount lost from illuminated retina is only 1.4% of the level of the tissue (corresponding to 0 meq K/gm N or about 0.3 mM of K-c centration) (Sekoguti, '60b). Also decrease in the K-level when the act transport of K-ion into the tissue is hibited by illumination is only about 5 r (Sekoguti, '60a). In addition, it is diffic to establish how high the initial K-leve maintained in the retinal tissue. On assumption that the loss of K-ions un illumination may stimulate the ATP activity of the retina, an attempt was

ed to examine if this activity changes connection with the changes in the K- l of the retina. Table 3 shows the re- s concerned. It is found that the yme activity becomes lower when the ner K-level is brought about by incuba- with glucose and glutamate. This fact gests that the fluctuation in the K-level he retina results in the changes in the centration of K-ions participating in ATPase activity.

he effect of K-ions on the ATPase ac- y is observed in myosine A or B atanabe et al., '53; Maruyama, '54; ven and Gershfeld, '57). Skou ('57) estigated the Mg-activated ATPase in microsomal fraction obtained from the nerves of shore crab, *Carcinus maenas*. ording to his results, Na-ions increase enzyme activity in the presence of Mg- s, while K-ions in high concentration ibit that part of the activity which is to Na^+, although K-ions increase the vity when the system contains both and Na-ions. In the present experi- nts, the mechanism of the effect of K- Na-ions is not yet fully understood, from the finding that the K-level of the na becomes higher in correspondence h the lowering of the Na-level (Sekoguti, a), it may be assumed that there occurs ne change in the relative concentration K- and Na-ions participating in the en- ne activity and this change results in the ivation of the ATPase in the retina. In w of this assumption it may be reason- e to consider that the movements of K- s can mediate the effect of illumination ch changes the ATPase activity of the na.

t is difficult to establish the site for the Pase activity in the retina. However, effect of illumination on the ATPase ivity is supposed chiefly due to the Pase activity of r.o.s., because the latter lso increased by illumination and this ct is evidently larger than the effect on enzyme activity of the whole retina ble 5).

According to Hara and Hara ('58), dopsin molecules, when illuminated, bably release monovalent cations which ve for the generation of impulses in on. On the other hand, the measurable ontent is found in the r.o.s. (0.1 meq K/gm N) and this content decreases rapidly with the lapse of time. It is assumed, therefore, that the release of K-ions from the r.o.s. is induced by illumination and accordingly the activity of the Mg-activated ATPase in r.o.s. may be accelerated. Thus the activity of the Mg-activated ATPase may be increased in connection with the potential generation in r.o.s. and the free energy liberated from ATP may serve for the restoration process.

Abood and Gerard ('54) supposed that the Mg-activated ATPase localized in the microsome found in the Schwann cell of the peripheral nerve may participate in the "machinery" function of ion transport, not in the "energy source," because this enzyme activity serves for the depression of the ATP-level. Skou ('57) offered also the hypothesis that the Mg-activated ATPase may participate in the active transport of ions. However, it is not yet determined in these experiments if the enzyme activity changes in connection with the nervous excitation. In the present experiment, one can establish at least the effect of illumination on the ATPase activity in the retina. Nevertheless, it is a subject to investigate in the future whether the ATPase activity estimated in the present paper plays a role in ion transport or is concerned with the restoration of the process participating in the generation or propagation of impulses.

SUMMARY

Using the tissue homogenate and the suspension of rod outer segments, the ATPase activities of cattle retina were determined. The obtained results are as follows.

1. The retinal homogenate shows the activity of the Mg-activated ATPase similar to the enzyme found in the microsomal fraction of muscle and nerve in view of the effect of Mg- and Ca-ions and of various inhibitors.

2. The suspension of rod outer segments reveals the same activity, though there are some differences from that in the tissue homogenate in respect to the effect of Mg- and Ca-ions and of inhibitors.

3. The enzyme activity depends on the concentrations of K-ions in the reaction medium; the maximum activity is found at 3.85×10^{-3} M (tissue homogenate) or

38.5×10^{-3} M (rod outer segments suspension) of K-concentration. The activity depends also on the change in the K-level of the tissue, as it becomes lower when the higher K-level is brought about by incubation with glucose and glutamate.

4. The activity of the Mg-activated ATPase is influenced by illumination of the retina. The activity in the tissue is slightly accelerated by illumination; in the rod outer segments more considerably.

5. Since the ATPase activity in the retina depends not only on the effect of light but also on the concentration of K-ions and moreover the K-level of retina is depressed through the loss of K-ions induced by illumination, the effect of illumination on the enzyme activity can be explained as being mediated by the movements of K-ions.

ACKNOWLEDGMENTS

I wish to express my sincere thanks to Professor Ichijiro Honjo for constant direction and valuable criticisms in the course of the work and in the preparation of the manuscript. My thanks go also to Mr. I. Koizumi and Mr. K. Nagai for their cooperation in the experiment.

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ensory Discrimination Between Polarized Light and ght Intensity Patterns by Arthropods[1]

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Directional orientation in the presence linearly polarized light has been amply monstrated in many different kinds of hropods both in the field and in the oratory. Discovered by von Frisch ('48,) in the honey bee this behavior has en reviewed in detail elsewhere (von sch and Lindauer, '56; Pardi, '57; ckhammer, '59 and Waterman, '59, b, d).

At least certain of these polarized light ponses are explicable only if the animals ncerned can see the direction of vibran of polarized light (which for conience will be referred to the *e*-vector en though the mechanism of detection not known) as distinct from other opal characteristics of the environment ch as intensities and wavelengths (von sch, '48; Autrum and Stumpf, '50; wles, '50; Pardi and Papi, '53a, b; Papi, ; Birukow and Busch, '57; Görner, '57; nder, '57; Lüdtke, '57; Jacobs-Jessen, ; Moody and Parris, '60; Waterman, a).[3] This means that a particular sen- y mechanism for polarized light must present (Autrum and Stumpf, '51; dtke, '57; Burkhardt and Wendler, '60). Nevertheless, the alternative explana- n has been proposed that most or all ectional responses in the presence of arized light depend merely on the same sory mechanism used in perceiving exnal light intensity patterns and not on specific visual polarization analyzer aylor and Smith, '53;[4] Stephens, Fingern and Brown, '53;[4] Bainbridge and terman, '58; Baylor and Smith, '58; mus, '58, '59; de Vries and Kuiper, '58; ylor, '59a, b; Smith and Baylor, '60). This alternative is supported by the owing two facts. First, differential rection, refraction and scattering of polarized light by the environment or by dioptric elements of the eye (Waterman, '54) can produce light intensity patterns with quadrants of maximal and minimal luminous intensity, the position of which is determined by the direction of the *e*-vector. Second, practically all arthropods do respond to light intensity patterns and can be expected to react to those caused by polarized light (Bainbridge and Waterman, '58; Smith and Baylor, '60; Waterman, '60a).

A major objective of the research here reported was to help resolve the contradictions involved in these two points of view by making a detailed study of polarized light and light pattern orientation in a variety of arthropods. The research was in this respect an extension of the study

[1] Aided by Contract Nonr 609(23) between the Office of Naval Research and Yale University, by grant 9690 from the National Science Foundation to Yale University and by an NSF grant-in-aid from the Bermuda Biological Station (Contribution no. 276).

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[3] Other parameters of polarized light which might be analyzed by an animal are: (1) degree of polarization, (2) type of polarization (linear, elliptical or circular) and (3) orientation of the major axis and ellipticity (for elliptical light). Most experiments, including the present ones, have been limited to testing the effects of different positions of the plane of polarization for nearly 100% linearly polarized light. Practically all polarized light in nature is linear (Ivanoff and Waterman, '58a,b; Waterman, '58), and this type is the only one so far known to affect animals differently than unpolarized light.

[4] The mechanisms suggested for reflection and refraction of linearly polarized light by ocular elements to establish intensity patterns responsible for *e*-vector orientation were different and apparently contradictory in the three cases analyzed: *Drosophila* (Stephens, Fingerman and Brown, '53), *Daphnia* and a water mite (Baylor and Smith, '53).

already begun on the interaction between horizontal light intensity patterns and responses to polarized light by aquatic arthropods (Waterman, '60a). Comparable studies on honey bee orientation have recently been published (von Frisch, '60; von Frisch, Lindauer and Daumer, '60). In addition, our experiments were designed to provide further information on the various kinds of orientation shown in the presence of vertically incident polarized light, particularly the directions of the basic orientation.[5]

METHODS

In previous experiments light intensity patterns have been altered by mirrors (Santschi, '11, '23; Jander, '57; Jacobs-Jessen, '59), by differential surface reflection (Baylor and Smith, '58; Kalmus, '58; Baylor, '59a, b; von Frisch, Lindauer and Daumer, '60; Smith and Baylor, '60) and underwater by differential scattering of the vertical beam of polarized light itself (Baylor and Smith, '53; Bainbridge and Waterman, '58; Waterman, '60a).

In the present experiments horizontal light intensity patterns were established by illuminating the sides of the experimental vessel. This allows a wider range of intensity patterns than the scattering technique and also permits the antagonistic and synergistic testing of polarization and intensity patterns.

To increase the generality of possible conclusions adults of 5 experimental species were chosen from three major classes of Arthropoda (table 1).

The *Daphnia* came from laboratory cultures and were identified with the aid of Brooks' ('57) monograph; the *Hyalella*, *Arrenurus* and *Bidessus* came from Linsley Pond, North Branford, Connecti and *Mysidium* from Walsingham P (marine), Bermuda.

Most of the animals were studied by method described under A below. measurements on *Mysidium*, carried ou the Bermuda Biological Station, were tained in the somewhat different way scribed under B.

A. General

Basically the experimental setup procedure were similar to those previo employed (Bainbridge and Waterm '57). The experimental vessel was a inder of transparent plastic 11 cm in ameter and 5 cm in height, contai about 3 cm of unfiltered pond wa Shielding this test area were a p screen surrounding the side walls of vessel, a 7 cm circular diaphragm cent on top of it and a 5.5 cm diaphragm be In this situation the depolarizer, w acted as the apparent light source vis to the experimental animals swimmin the transparent container, subtended angle of about 33°. The directional or tation of 15–20 animals was recorded f below at frequent intervals.

Although a meniscus was present in test vessel the top diaphragm shielded

[5] Basic orientation is a series of pure resp to external stimuli. Menotactic orientatio volves in addition internally initiated tu tendencies which cause deviations away fro basic directions. The experimental distin between them is that basic orientation is affected asymmetrically by changes in stim intensity whereas in menotactic orient changes in stimulus intensity modify the tional response asymmetrically (von Holst Mittelstaedt, '50; Jander, '57, '59, '60; S hammer, '59; Waterman, '60a).

TABLE 1

Experimental animals

Class	Subgroup	Species
Crustacea	Cladocera	*Daphnia pulex* (De Geer)
	Mysidacea	*Mysidium gracile* (Dana)
	Amphipoda	*Hyalella azteca* (de Saussure)
Arachnida	Acari	*Arrenurus* sp.[1]
Insecta	Dytiscidae (Coleoptera)	*Bidessus flavicollis* (Le Conte)

[1] Two closely related forms, *A. marshallae* (Piersig) and *A. megalurus* (Marshall) were used indiscriminately. These may be two species, but their exact systematic status is unsettled (Cook, '54).

side walls and the meniscus from the ical beam. This minimized the possi- y of reflection-refraction intensity pat- s from these sources. In different ex- ments the two diaphragms and the screen were either opaque black paper semi-translucent white paper. These e used in various tests with the follow- combinations of illumination (figs. 1 2, table 2). Condition 4, although not rted below in the results, was a con- to make certain that no orientation ferences were present in unpolarized t.

rimary illumination was a vertical m of linearly polarized (unpolarized control) light from a low voltage tung- optical bench projector emitting a rly parallel white beam. This passed cessively through a heat filter, a waxed er depolarizer and then a rotatable po- zing filter before reaching the experi- ntal vessel. The differential horizontal ttering of this polarized light in the water sets up a sinusoidal pattern of intensities with maxima at 90° to the *e*-vector and minima parallel (0°) to it (fig. 1, table 2). Oblique reflection from the bottom of the experimental vessel establishes a comparable pattern which was found to be essentially the same as that produced by horizontal scattering and reflection. This is true for the oblique bottom reflection pattern whether it is caused by the scattering of polarized light or by artificial horizontal light patterns.

To produce an unpolarized light source with the same geometrical relations and intensity (23,000 candelas (cd)/m²) depolarizer and polarizer were interchanged in the optical pathway.

Horizontal light patterns were established by having the two diaphragms and side screen made either of opaque black paper or of semi-translucent white paper. To mimic the light and dark sectors caused by directional scattering in the medium in the case of the white paper, supplementary

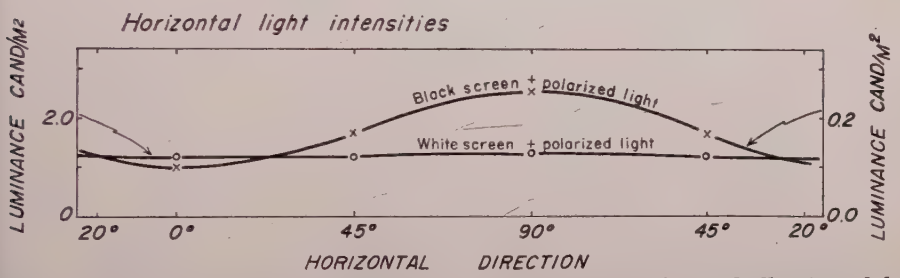

Fig. 1 Horizontal luminance distributions in the experimental vessel illuminated by a vertical beam of linearly polarized light (23,000 candelas/m²) and surrounded with either a black (Condition 1, table 2) or white screen (Condition 2, table 2) and diaphragms. Note the ordinate scale for the black screen condition (right) is only one tenth that for the white (left).

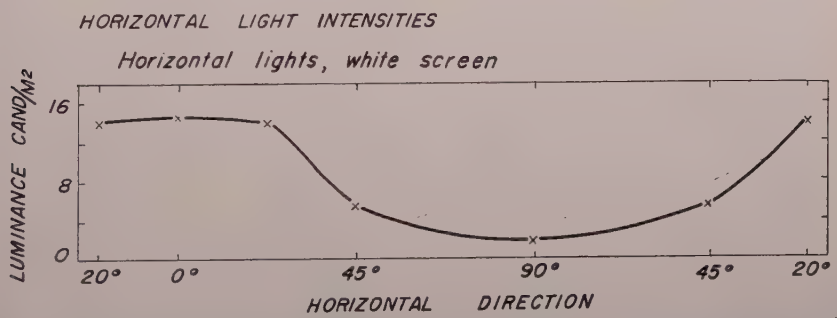

Fig. 2 Horizontal luminance distribution in the experimental vessel illuminated from outside by two opposed horizontal lights (Condition 3, table 2). This horizontal light intensity pattern should be compared with that produced by the scattering of polarized light (fig. 1) and that produced by black and white quadrants plus polarized light (fig. 6D).

light sources consisted of two horizontally directed microscope illuminators set 180° apart. The resulting luminances in various parts of the animals' visual field were photographed and then measured wi calibrated film (fig. 2, table 3).

In comparing the scattered light ir sity pattern with the artificially prod

TABLE 2

General illumination conditions

Stimulus conditions	Vertical beam	Horizontal lights	Side screen and diaphragms	Horizontal luminances		
				Max.	Min.	Av.
				candelas/m²		
1	Polarized	None	Black	0.26	0.10 (fig. 1)	0.18
2	Polarized	None	White	1.33	1.25 (fig. 1)	1.29
3	Unpolarized	Opposite quadrants illuminated	White	14.60	1.90	6.5 (fig. 2)
4	Unpolarized	None	White	—	—	1.29
5[1]	Polarized	Opposite quadrants illuminated	White	14.64	1.86	—

[1] For this condition the horizontal illumination was maximum in the directions in which the scattering of polarized light by the medium was greatest. Hence the phototactic effects of these components of the intensity pattern would act together.

TABLE 3

Mysidium illumination conditions

Stimulus conditions	Vertical beam	Horizontal lights	Side screen	Horizontal luminances	
				Max.	Min.
				candelas/m²	
1	Polarized	None	Black	0.76	0.34 (fig. 6D)
2	Polarized	None	White	7.7	6.5
3	Unpolarized	None	Black	—	—
4	Unpolarized	None	White	—	—
5	Unpolarized	White quadrants illuminated	Black and white quadrants	50.2	7.38
6	Polarized parallel to white quadrants	White quadrants illuminated	Black and white quadrants	50.0	1.48 (fig. 6D)
7	Polarized parallel to white quadrants	None	Black and white quadrants	3.4	0.93
8	Unpolarized	None	Black and white quadrants	White 3.6	Black 0.83
9	Polarized perpendicular to white quadrants	None	Black and white quadrants	3.8	0.73

e (figs. 1 and 2, table 2) note that direc-
nal differences in scattered intensities
re less than 0.2 cd/m². When supple-
entary horizontal lights were used, di-
ctional differences were 12.7 cd/m². The
aximum-to-minimum ratios were 3–7
nes larger with the artificial pattern, too,
ile the scattered light ratios were
ongly influenced by whether the back-
ound was black or white: max/min
tio for artificial pattern, 7.7; for scat-
ed light white surround 1.03, and black
rround 2.6.

The experimental procedure typically
as: (1) to take a series of 5 successive
otographs of directional orientation at
tervals of several seconds, (2) to change
e optical conditions in randomized order,
) to wait two minutes, (4) to take
nother series of 5 exposures and so on
til an adequate sample had accumu-
ed for each condition.

Where appropriate, the polarization
ane and the experimental vessel were
tated through 90° in successive series
der the same optical condition; this
uld control any undetected asymmetries
the vessel or in the illumination pattern.
nce stimulus conditions were the same
the two semicircles, opposite values
uld be combined, which doubles the
mber of measurements available in
0°.

Orientation angles were measured on
ojected images of the original films, and
e data plotted for 5° groups. Since the
ethod used did not provide samples with
actly the same numbers of observations,
sults have all been graphed as percent-
es against an abscissa representing
imuth angle. The resulting relation be-
een percentage observations and direc-
n of locomotion will be referred to as
orientation response curve for a given
perimental condition. Where statistical
sts have been made, differences having
values of greater than 1% for deviations
m the null hypothesis have not been
cepted as significant.

B. *Mysidium*

There were some optical differences
m the above setup in the tests on the
sid. The experimental vessel (18.5 cm
diameter) and diaphragms (10–12 cm
in diameter) were larger than in the other tests which is appropriate in view of the larger size of *Mysidium* (about 5–10 mm in length); also black and white paper quadrants were used to produce horizontal light patterns as in some earlier experiments (Baylor and Smith, '53; Bainbridge and Waterman, '58). For certain runs horizontal lights illuminated the white quadrants from outside. In this case their luminance observed from within the vessel was the sum of their reflectance of the vertical beam and their transmittance of the horizontal ones. The overhead vertical illumination had an apparent source (the depolarizer) subtending an angle of about 35° from the crustacean's point of view and had a luminance ranging from 15,000 cd/m² at its center to 890 cd/m² peripherally.

The following combinations of optical conditions were used in the various *Mysidium* experiments (table 3). Note that as in the general case (table 2, fig. 1) these horizontal luminance distributions (fig. 6D and table 3) show strong absolute and relative differences between the scattered light pattern and the various artificial ones. Conditions 3 and 4 although not reported below in the results, were controls to make certain that no orientation preferences were present in unpolarized light.

RESULTS

A. *Daphnia pulex*

Two main experiments and several controls were run on this cladoceran. In the first experiment the effects of polarized light were compared with those of horizontal light patterns. With the overhead vertically incident light beam linearly polarized and the black screen and diaphragms (Condition 1, table 2), *Daphnia* showed strong swimming orientation perpendicular to the polarization plane (figs. 3A and 4A). This was to be expected on the basis of the widespread occurrence of such responses among Cladocera (Baylor and Smith, '53; Eckert, '53) recently studied more exactly in relation to scattered light patterns in *Daphnia schødleri* (Waterman, '60a). In the present data the orientation response curve was sharply peaked (within 5°) perpendicular to the *e*-vector, and

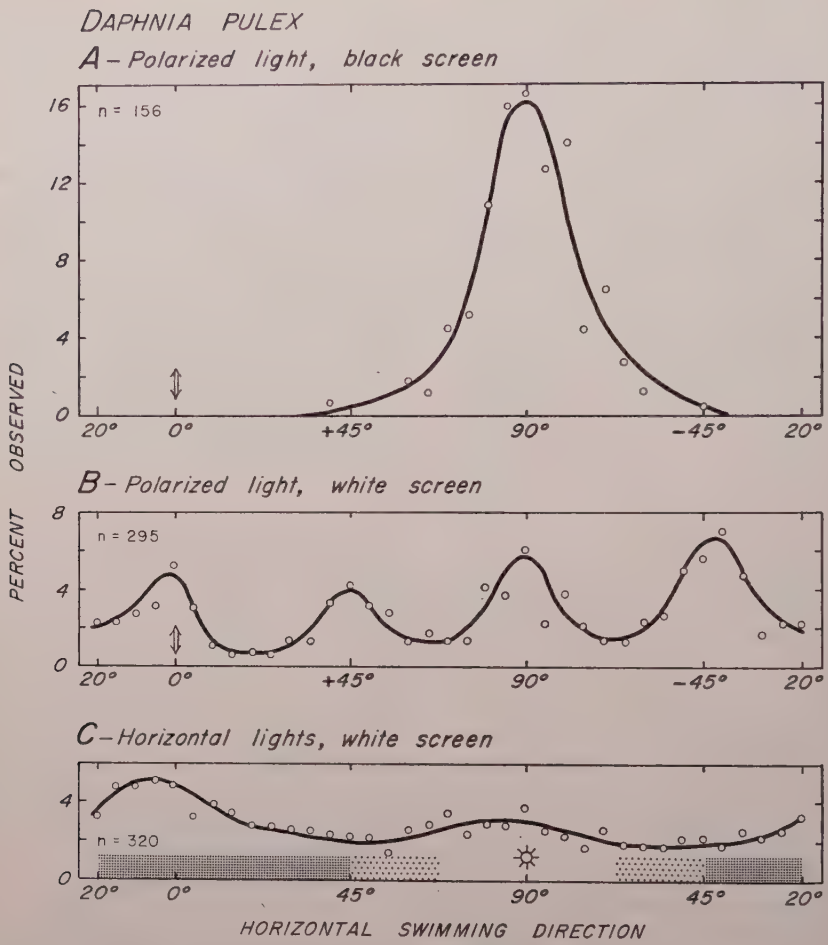

Fig. 3 Orientation of *Daphnia* under three conditions of vertical and horizontal illumination. A, vertical beam linearly polarized; black screen and diaphragms used (Condition 1, table 2). B, vertical beam linearly polarized; white screen and diaphragms used (Condition 2, table 2). C, vertical beam not polarized; horizontal light illuminating opposite quadrants, white screen and diaphragms used (Condition 3, table 2). The light intensity distribution produced by the horizontal lights is symbolized by the stippled shading along the abscissa in C. Further details of all the illumination patterns are given in figures 1 (3A, B) and 2 (3C). Each graph shows the relative numbers (%) of animals observed to be swimming in the direction indicated, summed within 5° intervals between 0° (parallel to the *e*-vector of the polarized light) and 90° (perpendicular to the *e*-vector) right (+) and left (—) of the *e*-vector. As far as stimulus conditions go, this semicircle is symmetrical with the opposite one. The position of the *e*-vector, when polarized light is present, appears as a vertical double headed arrow while the direction of the supplemental horizontal illumination, when present, appears as a circle with radiating rays. The number of measurements for each graph is shown as *n* at the left. The open circles are plots of the actual data points while the solid lines are smooth curves fitted by eye to these observations. Where these orientation response curves show significant peaks and valleys, as in all cases plotted, swimming directions are not random and the peaks in the curves represent preferential directions of swimming. The presence of 4 peaks (B) is considered basic for a vertical beam of polarized light, a single peak at 90° represents a particular kind of altered response. The two peaks in C indicate the presence of both positively and negatively phototactic individuals, which behaved alike in polarized light (A and B).

individuals were recorded swimming
ore than 50° from perpendicular.
In contrast two minor response peaks
re shown by the same population of
phnia pulex with the overhead verti-
lly incident light beam depolarized and
e white screen illuminated in opposite
adrants (Condition 3, table 2; fig. 3C).
e major peak near 0° indicates that
ost of the animals were negatively photo-
ctic, swimming towards the two dark
ctors of the horizontal light pattern.
owever, the minor peak in the lighter
ctors proves that others were positively
ototactic. The presence of both posi-
ely and negatively phototactic individ-
ls was confirmed in the vessel with the
ack surround by using an oblique test
ht. Hence the black screen was
t suppressing negatively phototactic re-
onses.
This experiment shows that *Daphnia*
th both positive and negative phototactic
ns were present under the two condi-
ns. Yet no animals were found swim-
ng parallel to the polarization plane
th the black screen as negative individ-
ls would do if differentially scattered
ht were the effective stimulus for orien-
ion to the *e*-vector. Therefore the
ientation perpendicular to the polariza-
n plane must be a specific response
dependent of intensity pattern.
In the second experiment with the same
pulation of *Daphnia* the effects of black
reens and diaphragms were compared
th white ones when polarized light was
ed in the vertical beam for both condi-
ns. With the white surround (Condition
table 1) *Daphnia* orients quite differ-
tly (fig. 3B) than it does with the black
g. 3A). Instead of one preferred swim-
ng direction with the white diaphragms
d screen there are 4 at 0°, ±45° and
° relative to the *e*-vector.
These 4 basic swimming directions
uld be shown statistically to occur with
gnificantly different frequencies (fig.
). Three replications of this experiment
th different groups of *Daphnia* revealed
e same 4 modes in their orientation. A
test was made to determine whether the
ngle orientation direction observed with
e black surround was qualitatively dif-
rent from the 4 directions shown at lower overall intensities and higher horizontal intensities. To do this 4 distribution peaks each like the single peak but appropriately reduced in height were superimposed on the real 4-peak curve. No significant difference in form was present between the observed 4-peak curve and the one produced in this way. Hence equal orientation accuracy is shown for each of the 4 basic directions and for the black and white surrounds. Comparable conclusions are reached below for *Mysidium* (p. 147).

Selection of individual *Daphnia* orienting in one of the 4 orientation angles yields a group showing all 4 preference directions. Hence a single animal must be able to orient its swimming in any one of these 4 directions.

Three possible explanations could account for the difference in *Daphnia's* orientation responses with the black and white screens; control experiments on a single population of animals were carried out to determine which were actually involved. First, the generally lower light intensity arising from absorption by the black screen and diaphragms might be responsible for the single direction of response with the black surround. This was tested by substantial light intensity reductions both with black and white surrounds (Conditions 1 and 2, table 2).

Reducing the vertical polarized beam successively to 10% and 1% of its original intensity evoked no detectable change of behavior with the white screen (fig. 4C and D). However, with the black screen, intensity reduction to 10% and 1% caused the appearance of 4 orientation peaks so that at the lowest intensity the behavior pattern was closely similar to that previously observed only with the white surround (fig. 4A and B). This influence will be referred to as the *intensity effect*. Since it is just opposite the effect predicted if the single peak with the black screen resulted from low light intensities, such an explanation must be rejected.

Second, light intensity patterns due to directional scattering by the medium or to differential reflection by the side walls or diaphragms might possibly be more effective with the black screen and thus account for the different behavior with black and

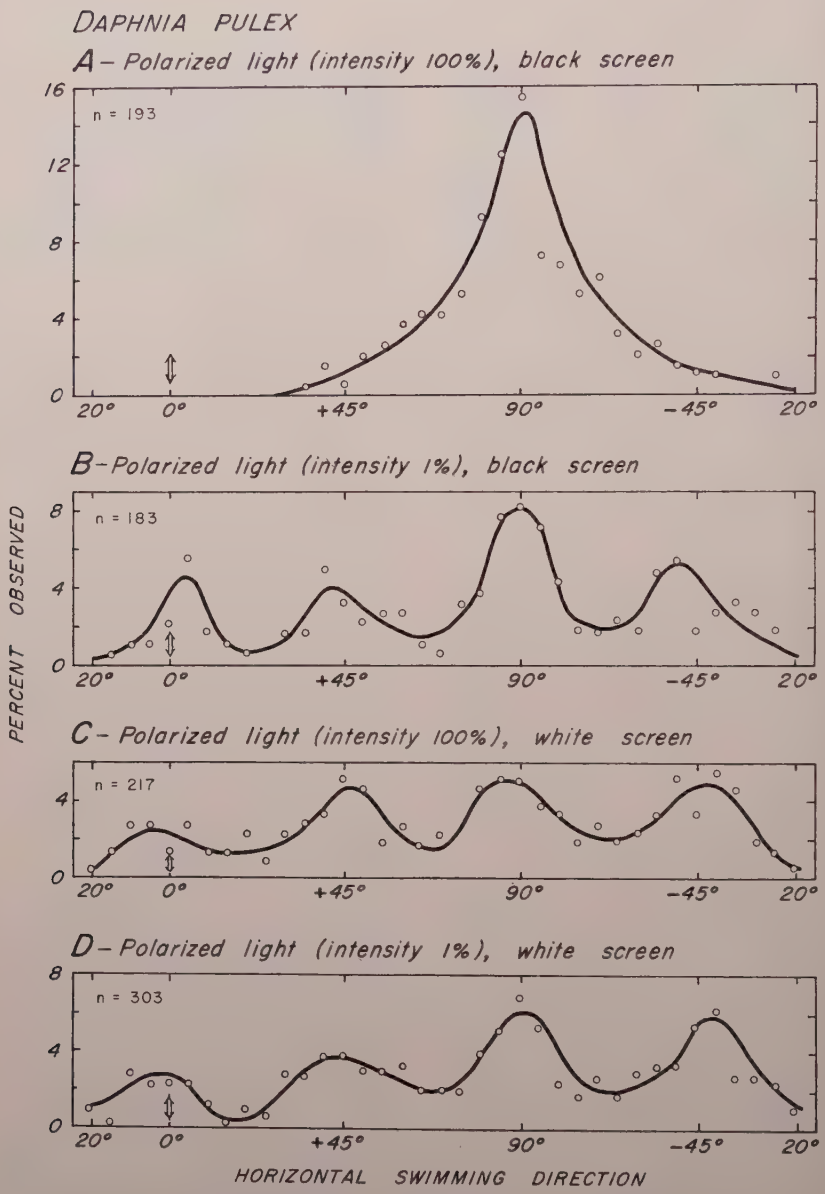

Fig. 4 Orientation of *Daphnia* under 4 conditions of vertical and horizontal illumination. Further details of the illumination patterns for A (Condition 1, table 2) and C (Condition 2, table 2) are given in figures 1 and 2. In B (Condition 1, table 1) and D (Condition 2, table 2) a Wratten neutral filter of optical density 2.0 was placed in the optical pathway. Hence the light intensity patterns were the same respectively as A and C, but the overall intensity was reduced to 1%. Note that at full intensity changing from white (C) to black (A) screen causes the animals to orient only perpendicular to the plane instead of in all 4 basic directions. At the lower overall intensity level (B, D) this reaction did not occur. Symbols and details of the graphic method are explained further in the legend of figure 3.

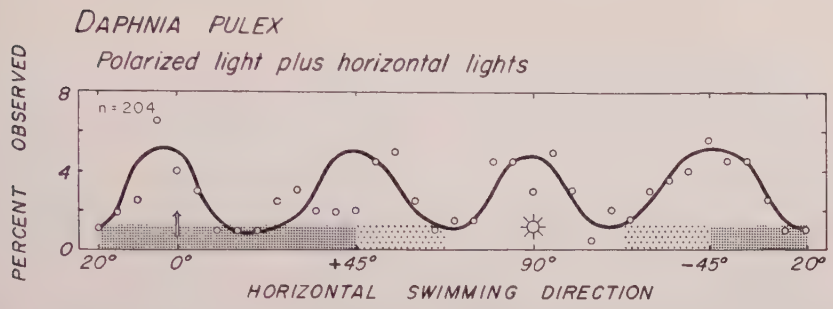

Fig. 5 Orientation of *Daphnia* when the vertical light beam is polarized and an artificial pattern of horizontal light is produced by two lights 180° apart located at 90° relative to the *e*-vector (Condition 5, table 2). Symbols and details of the graphic method are explained further in the legend of figure 3. Note that even though the artificial horizontal light intensity maxima are centered in the same direction as the maximum differential scattering of polarized light that 4 nearly identical peaks in the response curve indicate unequivocally the 4 basic directions of swimming relative to the *e*-vector of polarized light.

ıite surrounds. However, setting a hori-ıntal illumination pattern in phototactic mmation to such a scattering pattern ondition 5, table 2; fig. 5) does not af-ct the presence of 4 orientation peaks th the white surround. Consequently is second hypothesis must also be re-ted.

This leaves as the only possible remain-g explanation the difference between the minance of the side walls and dia-ragms and that of the overhead light ırce which is less with the white screen ın with the black. The suppression of ee of the 4 basic swimming directions d the switching of all orientation to the ° direction when this difference is large ough will be referred to as the *light con-st reaction.* It appeared when the hori-ıntal illuminance was about 0.19 cd/m² th the vertical beam at 23,000 cd/m². is reaction demonstrates some sort of age perception in *Daphnia.* The inten-y effect is thus seen to be an influence the light contrast reaction and appeared the present experiments with smaller ferences between horizontal and vertical ımination: 1.3 cd/m² horizontal lumi-nce, 23,000 cd/m² vertical.

B. Mysidium gracile

The basic experiment done on this ani-l was a comparison of polarized light ponses with light intensity responses; veral replications of the same experi-nt were made on different populations d the results combined. With the verti-cal light beam polarized and the black surround (Condition 1, table 3; luminance distribution shown in fig. 6D), *Mysidium,* like *Daphnia,* showed only one preferential swimming direction perpendicular to the *e*-vector (fig. 6A). This agrees with earlier measurements (Bainbridge and Waterman, '57, '58; Waterman '60a). Nearly 7% of the orientation were within 5° of the perpendicular and more than 30% were within 15°. However, some observations were recorded in all directions of swimming relative to the plane of polarization.

With the vertical light unpolarized, black and white quadrants and horizontal illumination of the white quadrants (Condition 5, table 3), the orientation pattern rather resembles that obtained with the polarized light beam (fig. 6B). There is one preferred and somewhat more marked orientation direction coincident with the white illuminated sectors (fig. 6D) although substantial records were obtained of animals swimming in all directions.

When the two previous conditions were added together so that the brightest horizontal quadrants coincide with the plane of polarization (Condition 6, table 3; luminance distribution shown in fig. 6D), two peaks appear in the orientation curve (fig. 6C). The larger of the two is in the sector parallel to the plane of polarization which coincides with the most luminous region of the side walls. The smaller of the two is at 90° to this. Observation of individual animals indicates that they can change

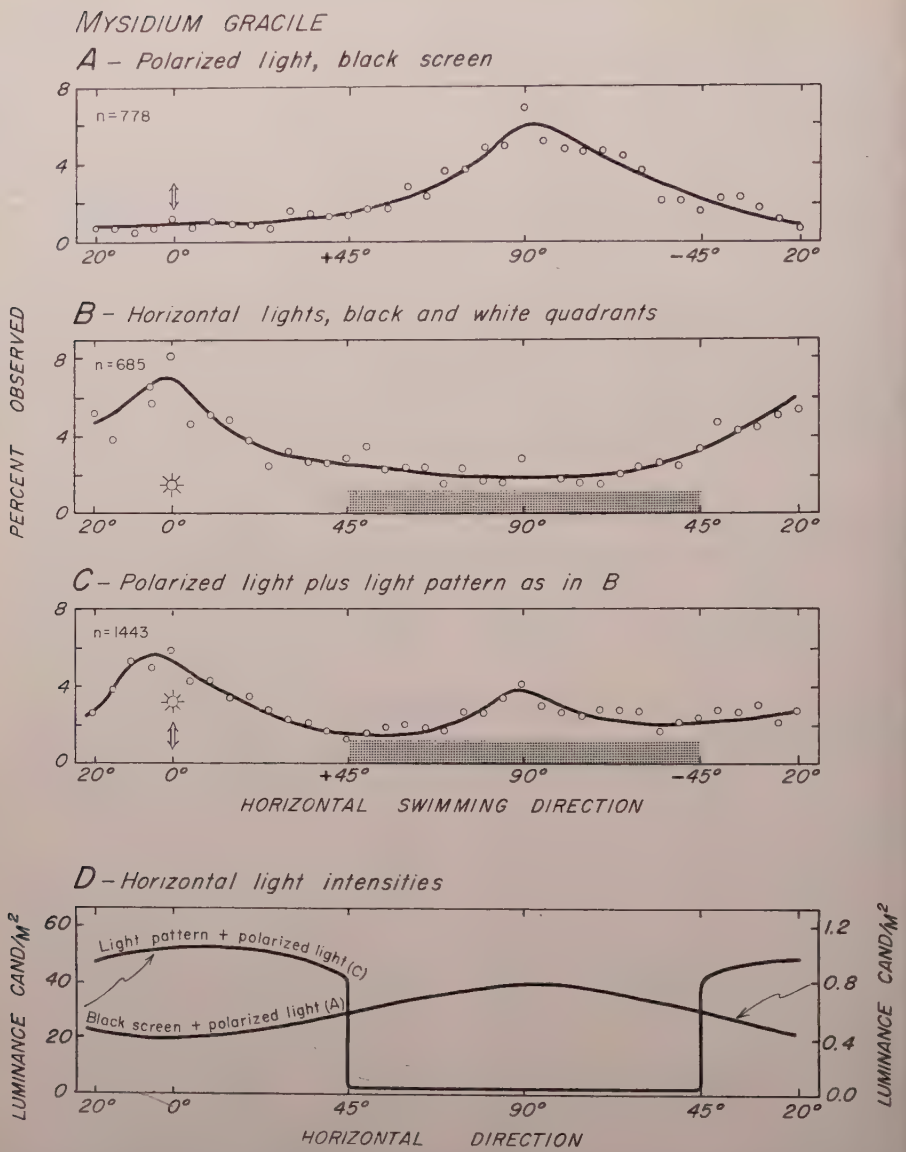

Fig. 6 Orientation of *Mysidium* (same individuals as in figure 7) to three conditions of vertical and horizontal illumination (A, Condition 1, table 3; B, Condition 5, table 3; C, Condition 6, table 3); light patterns specified further in D (Conditions 1 and 6, table 3). The position of the black quadrant is symbolized by the stippled shading along the abscissa in B and C. Symbols and details of the graphic method are explained further in the legend of figure 3. Note that despite the strong positively phototactic response indicated by the single peak in curve B, polarized light added to the artificial light pattern evoked in addition a second preferential swimming direction (C) in the center of quadrants of minimum light intensity.

from one of these preferential directions to the other. This change and similarity of responses to polarization and intensity patterns separately and together suggest that *Mysidium* may orient either to brightn pattern or to polarization plane.

If the horizontal illumination of white quadrants was eliminated (Co

n 7, table 3; fig. 7B) two preferential rections were still present, but the one rpendicular to the *e*-vector was the rger, reversing the relation in figure 6C. ow when the vertical beam is not polar-ed (Condition 8, table 3) the mysids ow a single preferred direction towards e white sectors but this is less prominent an when these are illuminated (fig. 6B; ndition 5, table 3).

When the intensity pattern and the larization act synergistically (Condition table 3), the orientation pattern has only e peak instead of the two present when ey act antagonistically at 90° to one an-ner. With the white screen (fig. 7A; Con-tion 2, table 3) most of the mysids swim rpendicular to the plane, but a signifi-nt preference is also shown for parallel ientation. However, peaks at ±45° to e *e*-vector have not yet been obtained th *Mysidium*. Differences between these sults and those with the dark screen are volved here.

Direct evidence for individual *Mysidium* anging their swimming direction from rpendicular to parallel is not available th polarized light and the white screen. owever, such a change can be demonstrated by going from a dark screen to a light screen which causes some individuals to alter their preferred orientation in that way. Comparison of the form of the peaks (by the method used for *Daphnia*, p. 143) in the unimodal and bimodal frequency distributions with the black and white screens respectively, shows that it does not differ significantly. Therefore, the increased horizontal luminosity only evoked a second preferred direction of swimming but did not qualitatively change the *form* of the response. This conclusion is comparable to that reached for *Daphnia* (p. 143).

C. *Hyalella azteca*

For this organism a comparison was made of its oriented responses to polarized light and to light intensity patterns. The oriented swimming of this amphipod was quite similar to that of *Daphnia pulex*. With the vertical beam unpolarized and with horizontal illumination of the white screen (Condition 3, table 2) a curve with two peaks was obtained (fig. 8A) with equal numbers of observations of positively and of negatively phototactic swimming orientation. The same populations of individuals responded to a polar-

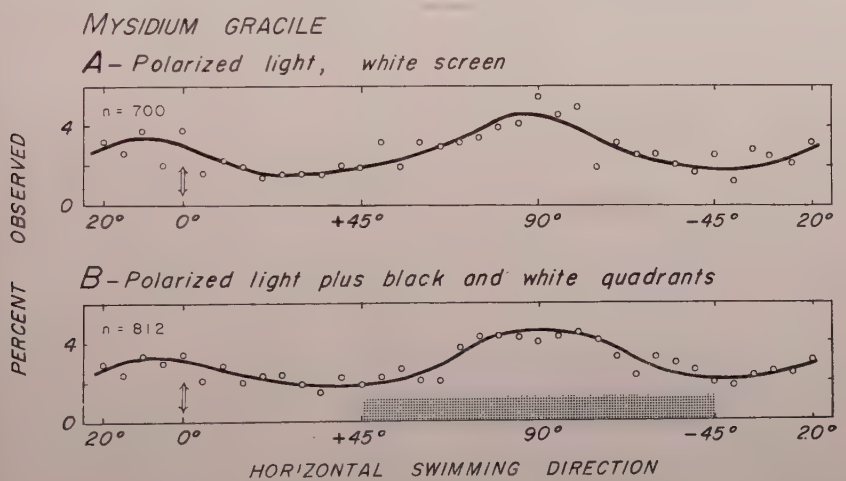

Fig. 7 Orientation of *Mysidium* (same individuals as in figure 6) to two conditions of vertical and horizontal illumination (A, Condition 2; B, Condition 7, table 3). The position of the black quadrant is symbolized by the stippled shading along the abscissa in B. Symbols and details of the graphic method are explained further in the legend of figure 3. Observe that the bimodal response curve to polarized light (A) is maintained when the surround consists of black and white quadrants so placed that in fact the major preferential direction is centered in the middle of the minimum intensity quadrants of the horizontal light pattern even though orientation to black and white quadrants alone (fig. 6B) demonstrates only a positively phototactic response.

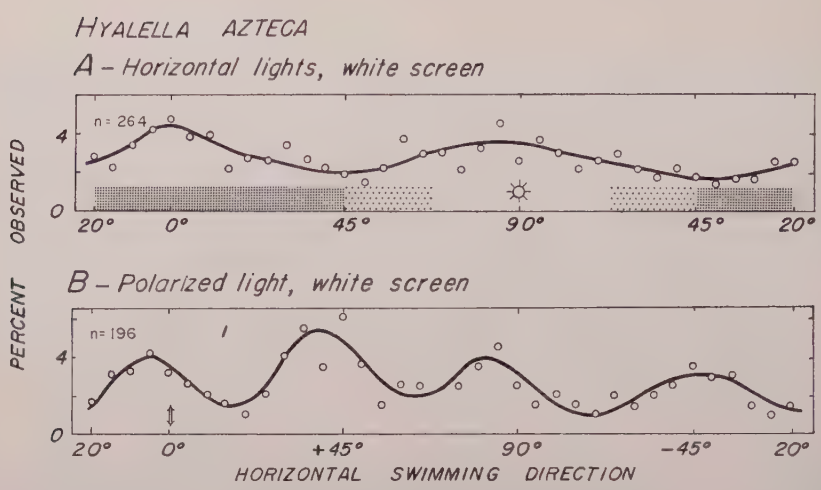

Fig. 8 Orientation of *Hyalella* to two conditions of vertical and horizontal illumination (A, Condition 3, table 3; B, Condition 2, table 2) specified further in figures 1 and 2. Symbols and details of the graphic method are explained further in the legend of figure 3. No sign of ± 45° orientation appears without the polarized light, although the bimodal curve in A is evidence for both positively and negatively phototactic responses. The 4 basic swimming directions appear with the vertical beam polarized and the screen and diaphragms white.

ized vertical light beam with the white screen (Condition 2, table 2) by showing 4 peaks in its orientation curve at 0°, ± 45° and 90° relative to the *e*-vector (fig. 8B). These 4 preferred directions of orientation were present both with black and white screens (table 4). The small increase which occurred at 90° with the black screen is not significant. Similar responses to polarized light were obtained on three replications with other groups of *Hyalella* individuals.

Unlike *Daphnia* and probably *Mysidium* no light contrast reaction was found for the amphipod.

D. Arrenurus sp.

With the white screen and with the vertical beam polarized (Condition 2, table 2) the mites showed 4 preferential directions of orientation as in *Daphnia* (fig. 9A). Four replications of this experiment wi the same population of animals gave sir lar results except for the fusion of t peaks in one case. With the vertical bea unpolarized and the white screen horizo tally illuminated in two sectors 180° ap (Condition 3, table 2), a weak positiv phototactic orientation was observed (f 9B).

Comparisons of the orientation sponses to the polarized light pattern w white and with black screens demonstrat that a light contrast reaction similar but weaker than that of *Daphnia* does cur in this water mite (table 5). The re tive frequency of orientation at 90° to t *e*-vector increased from 25.7% to 47.7 with the dark surround while an acco panying decrease occurred in the 45° rections. These differences between curves are statistically significant.

TABLE 4

Frequencies of basic directions in Hyalella

Side screen	*n*	0°	+45°	90°	−45°
White	223	17%	23%	25%	35%
Black	219	16%	27%	31%	26%

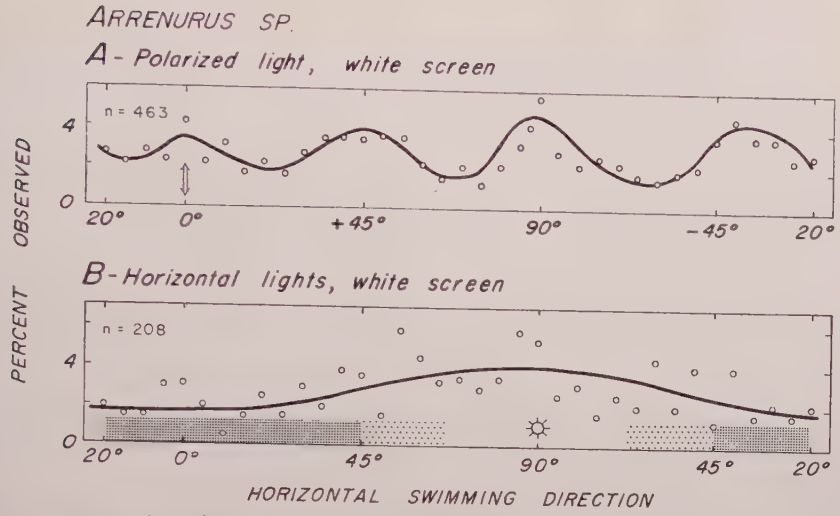

Fig. 9 Orientation of *Arrenurus* to two conditions of vertical and horizontal illumination (A, Condition 2; B, Condition 3, table 2) specified further in figures 1 and 2. Symbols and details of the graphic method are explained further in the legend of figure 3. Despite the weak positive phototaxis indicated by the unimodal response curve to the horizontal light pattern without polarized light (B) strong orientation in the 4 basic swimming directions is shown by the 4 well developed peaks with the white screen and vertical beam linearly polarized (A).

TABLE 5

Relative frequencies of basic directions in Arrenurus

Side screen	*n*	0°	+ 45°	90°	− 45°
White	136	12.5%	29.4%	25.7%	32.4%
Black	166	11.5%	19.6%	47.7%	21.7%

E. Bidessus flavicollis

Comparisons were made of the oriented imming responses of the same group of etles when they were positively photo-ctic and when they were negative. The imals were positive if light adapted and disturbed; negative responses were ob-ned by dark adapting for a day and itating the experimental vessel before e measurements.

If the polarized light and the horizontal ensity pattern were used together antag-istically (Condition 5, table 2) on posi-ely phototactic *Bidessus*, a bimodal entation curve was obtained. Most of e beetles swam in the most luminous tor of the horizontal intensity pattern, t a small preference was shown perpen-ular to the polarization plane in direc-ns of the least luminous side walls.

Positively phototactic animals show 4 rked preference planes with the vertical beam polarized and with the white screen (Condition 2, table 2; fig. 10A). Swimming perpendicular and parallel to the *e*-vector showed a slight predominance over the ±45° directions. With the vertical beam unpolarized and horizontal illumination of the white screen (Condition 3, table 2) these positive beetles showed strong orientation to the brightest sectors (fig. 10B). The maximum in the orientation curve near the luminance peak was strongly marked and few individual measurements were more than ±50° of this point. No signs of more than one preferential direction were present.

The response curve of the same animals when negative also showed a single orientation peak to the horizontal intensity pattern on the white screen, but in this case the preferential direction was towards the darker quadrants of this horizon, 90° from the luminance maxima of the side walls

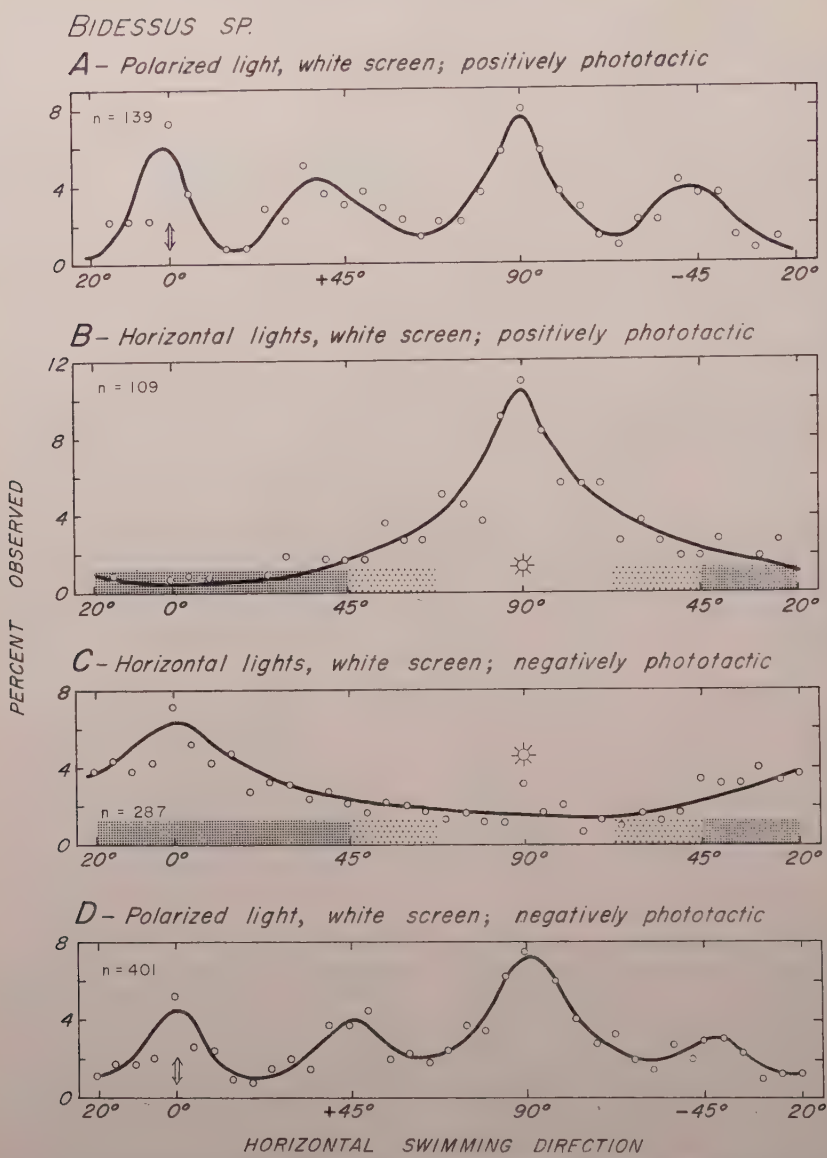

Fig. 10 Orientation of *Bidessus* under two conditions of vertical and horizontal illumination (A and D, Condition 2; Band C, Condition 3, table 2) specified in figures 1 and 2. Details of the graphic method are explained in the legend of figure 3. The comparison of the two illumination conditions was made twice, once with the population of beetles positively phototactic (A and B), and once with them negative (C and D). Observe that essentially identical orientation in the 4 basic swimming directions was obtained with polarized light with both conditions of the animal even though the phototactic responses were reversed.

(fig. 10C). Relative height of the peak in the orientation curve was not as great as in the positive case but recall that the beetles had to be disturbed to make them strongly negative. In sharp contrast, the negatively phototactic insects showed the same orientation responses as the positive ones v the polarized vertical beam and wl screen (fig. 10D). Thus changing ph tactic sign in this species powerfully al its orientation to intensity patterns in way expected but has no significant ef

the presence of the four peaks and eir relative weights in the orientation rve to polarized light.

DISCUSSION AND CONCLUSIONS

The various experimental data presented ove permit a number of definite conclu- ns to be drawn and at the same time ise certain problems which deserve some scussion. These fall mainly into 5 cate- ries: A, the problem of polarized light d light pattern vision; B, the occurrence, oice, and ecological significance of the basic steering directions; C, the mecha- sm of polarized light sensitivity; D, eaning of the light contrast reaction and tensity effect; and E, the question of cen- al integration of the polarized light sen- ry data.

A. Polarized light and light pattern vision

One important aspect of the present rk is its bearing on the problem of po- rized light vision as a distinct physiologi- l process. Ten different lines of evidence ove that a wide variety of arthropods ust see the plane of vibration of linearly larized light. Of this evidence 6 relevant ints can be made from our own data.

1. In *Daphnia* and *Bidessus* reversal of ototactic sign produces a corresponding versal in response to horizontal light in- nsity patterns consisting of two opposite ht and two opposite dark quadrants. Yet is change in phototactic sign has no ef- ct on basic orientation to the plane of larization (p. 143 and fig. 10). Hence eir polarized light orientation cannot be response to horizontal light intensity tterns of the sort used which resemble se produced by differential scattering d reflection of polarized light.

2. In all experiments testing horizontal tterns of light intensity, the animals ver showed more than two directions of imming, one towards the lighter quad- nts of their horizon and one towards the rker quadrants, corresponding respec- ely to positive and negative phototaxis gs. 3C and 8A). Nor has a persistent lique 45° course relative to a light stim- us yet been reported in the literature. In ntrast, up to 4 basic orientation direc- ns appear in a vertical beam of linearly polarized light. This difference in response implies that two distinct sensory mecha- nisms must be involved.

3. In many cases responses to the *e*-vector of polarized light showed smaller deviations from the most strongly pre- ferred direction than those to horizontal intensity patterns even though the ratios of maximum to minimum intensities arti- ficially produced were much greater than those resulting from directional scattering of polarized light (figs. 3B, 3C, 6A and 6B). This is the reverse of the behavior pre- dicted if both were responses to horizontal light patterns. Hence both reactions can- not be intensity responses.

4. *Mysidium,* in the presence of both polarized light and horizontal light pat- terns together, proved its ability to distin- guish between them by reacting character- istically first to one and then to the other as if each were present alone (pp. 145–146 and fig. 6C). Thus when maximal hori- zontal light intensities are artificially estab- lished in the direction of the *e*-vector of a vertical beam of polarized light then the animal swims perpendicular to the *e*-vector reacting to the polarization part of the time, and parallel to the *e*-vector respond- ing part of the time to the intensity pat- tern. Therefore, polarized light and inten- sity patterns are two distinct visual qual- ities in the animal's perception.

5. For *Mysidium,* if the dark quadrants due to scattering of the vertical beam of polarized light are artificially made more luminous than those where maximum dif- ferential scattering occurs, significant pref- erential orientation continued to be per- pendicular to the *e*-vector as it was before the artificial intensity increase. In other words, when the horizontal intensity pat- tern due to scattering is overcompensated by an artificial one so that the relative in- tensity of the four quadrants is reversed, preferential orientation 90° to the *e*-vector persists even though these quadrants are now darker than the ones parallel to the plane. Hence the polarized light response cannot be a positive phototaxis toward the brighter sectors. Comparable experiments yielding similar results were also done with *Daphnia* and *Bidessus* but these have not been described above.

6. For *Daphnia* the light contrast reaction, a response to differences between vertical and horizontal luminances, disappears completely at low overall levels of illumination, yet the orientation relative to the *e*-vector of polarized light shows no detectable deterioration (fig. 4). This makes it difficult to believe that the polarized light response depends on intensity ratios of horizontal illumination in various azimuths.

From the previously published data on terrestrial arthropods 4 kinds of experimental results can be cited which are only explicable if polarized light is perceived as such. These must be added to the list derived from our present experiments to obtain a proper summary evaluation of the problem.

1. In the honey bee orientation under a polarizer has the same angular relation to the *e*-vector that it has to the natural polarized light of corresponding parts of the blue sky despite great differences in intensity patterns under natural and artificial conditions (von Frisch, '48, '49).

2. Since bees know the regional distribution of polarized light in the sky (von Frisch, '48, '49), they must know the direction from which it is coming. Yet they cannot with ordinary image vision infer the direction of the original source from a light pattern established by reflection and refraction.

3. Under natural conditions reflection and refraction patterns due to polarized sky light must to a considerable extent cancel each other out because the plane of polarization is different in various parts of the sky. In addition such patterns as do arise will be confused by much more marked intensity patterns due to direct sunlight, which comprises up to 80% of the total sky light, and to clouds and surface details of the earth. To claim that bees could learn sky polarization patterns from these reflection-refraction cues observed on their field trips seems highly unlikely in view of such conditions.

4. In this vein, experiments with spiders (Papi, '55; Görner, '57), ants (Jander, '57) and bees (Jacobs-Jessen, '59), have shown in some instances that when the main intensity pattern is dislocated by transposing the sun 180° with a mirror, the animals nevertheless maintain their ori tation direction relative to the blue sky.

Further extensive proof that in honey bee polarized light sensitivity i distinct physiological process has recen been published by von Frisch ('60) a von Frisch, Lindauer and Daumer ('6

Altogether these facts demonstrate t responses to light intensity patterns c not be used to "explain" the polarized li orientation of arthropods as some work have tried to do (Baylor and Smith, ' '58; Stephens, Fingerman and Brown, ' Kalmus, '58; de Vries and Kuiper, ' Baylor, '59a,b; Smith and Baylor, '60). stead our data indicate that there must two distinct visual input channels, one polarized light vision and one for intens pattern discrimination. Combined with other data cited for terrestrial as well aquatic arthropods, the total relevant dence now available suggests that the sa conclusion may be drawn for most cru ceans, insects and at least some arachni

B. The 4 basic steering directions

A second matter which requires furt consideration is the presence in all our perimental animals, except *Mysidium*, 4 preferential directions of orientation the plane of polarized light under cert conditions. Orientation at ± 45° to *e*-vector was first reported for the be *Geotrupes sylvaticus* by Birukow ('53) the possible significance of this find was not discussed; similarly Jacobs-Jes ('59) found 45° obliquely polarized li orientation in the solitary bees *Andr* and *Halictus* as well as the ant *Form* The directions of basic orientation to *e*-vector of polarized light now known various arthropods are shown in table

In view of the demonstrated occurre of 4 basic steering angles relative to *e*-vector of polarized light in branchio and peracaridan crustaceans, in a wa mite and in at least two orders of inse it is tempting to consider that all art pods (at least those with compound ey have this same basic orientation wit preferential directions. One might ob that the lack of evidence for perpendicu or parallel orientation in *Andrena* or failure to find ± 45° orientation in m of the earlier work would provide m

TABLE 6

Occurrence of basic steering directions in polarized light

Animal	0°	90°	0°, 90°	+45° −45°	0°, +45° −45°, 90°	Reference
CRUSTACEA						
Anostraca						
Artemia	0	+[1]	0	0	0	Stockhammer, '59, unpub.
Cladocera						
Bosmina	0	+	0	0	0	Baylor and Smith, '53
Ceriodaphnia	0	+	0	0	0	Baylor and Smith, '53
Chydorus	0	+	0	0	0	Baylor and Smith, '53
Daphnia	0	+	0	0	0	Baylor and Smith, '53
Daphnia	0	+	0	0	0	Waterman, '60a
Daphnia	0	+	0	0	+	Jander and Waterman, '60
Kurzia	0	+	0	0	0	Baylor and Smith, '53
Leptodora	0	+	0	0	0	Baylor and Smith, '53
Moina	0	+	0	0	0	Baylor and Smith, '53
Sida	0	+	0	0	0	Baylor and Smith, '53
Simocephalus	0	+	0	0	0	Baylor and Smith, '53
Mysidacea						
Mysidium	0	0	+	0	0	Bainbridge and Waterman, '57
Mysidium	0	+	0	0	0	Bainbridge and Waterman, '58
Mysidium	0	+	0	0	0	Waterman, '60a
Mysidium	0	+	+	0	0	Jander and Waterman, '60
Isopoda						
Oniscus	0	0	+	0	0	Birukow, '56, unpub.
Porcellio	0	0	+	0	0	Birukow, '56, unpub.
Amphipoda						
Hyalella	0	0	0	0	+	Jander and Waterman, '60
INSECTA						
Diptera						
Drosophila	0	+	+	0	0	Stephens, Fingerman and Brown, '53
Mosquito larvae	0	0	0	0	(+?)	Baylor and Smith, '53
Coleoptera						
Bidessus	0	0	0	0	+	Jander and Waterman, '60
Geotrupes	0	0	0	0	+	Birukow, '57
Trichoptera larvae	0	+	0	0	0	Baylor and Smith, '53
Hymenoptera						
Andrena	0	0	0	+	0	Jacobs-Jessen, '59
Apis	0	0	+	0	0	Jacobs-Jessen, '59
Bombus	0	0	+	0	0	Jacobs-Jessen, '59
Camponotus	0	0	+	0	0	Jacobs-Jessen, '59
Formica	0	0	0	0	+	Jacobs-Jessen, '59
Halictus	0	0	0	0	+	Jacobs-Jessen, '59
Trigona	0	0	+	0	0	Jacobs-Jessen, '59
Vespa	0	0	+	0	0	Jacobs-Jessen, '59
CHELICERATA						
Xiphosura						
Limulus[2]	0	+	0	0	0	Baylor, '59
Acarina						
Arrenurus	0	0	0	0	+	Jander and Waterman, '60
Fresh water mite	0	+	0	0	0	Baylor and Smith, '53

+ = present, 0 = absent.

[2] Distinction between polarized light perception and light pattern perception not clearly established.

ceptions to the hypothesized general e.

However, our experience with *Daphnia* which oblique and parallel orientation, t previously observed despite a great deal of work on these animals, appear with dramatic force under appropriate experimental conditions (figs. 3, 4 and 5), should induce caution towards accepting negative evidence against this rule before a great

variety of experiments have been done on a given animal. With this reservation, well established exceptions to the general rule that 4 basic steering angles form the primary orientation pattern to polarized light do not exist at present.

Even though each of the 4 basic swimming directions must reflect a different physiological state, the similarity in the shape of the peaks in the response curves indicates that these are fundamentally similar (pp. 143 and 147).

The systematically widespread occurrence of the 4 basic orientation directions within the arthropods demands an explanation. Either evolutionary or adaptive explanations (Waterman, '60c; Waterman and Chace, '60) are the main kinds of interest here. However, the phylogenetic homology of this behavior pattern in the various arthropod groups cannot be finally determined from the nature of the eyes since their probable homologies are not yet well enough established.

From the point of view of ecological adaptation nothing is known about the advantage of having just 4 basic orientation directions. In all practical field cases like homing, menotactic rather than basic orientation is involved. Possibly the 4 primary steering angles are a component of the menotactic mechanism. With their aid deviations from the basic directions brought about by spontaneous turning tendencies need be no greater than 22.5° to make possible directed steering in any compass direction. This would in fact be menotaxis. Previous work has shown that deviations up to 30° from the basic steering direction may occur in *Daphnia* (Waterman, '60a).

Such evidence proves that it is physiologically reasonable to consider that menotactic orientation is elaborated from the basic orientation. This would be analogous to the relationship between basic and menotactic orientation in the case of steering with the aid of directional light sources (Jander, '57, '59, '60).

C. Mechanism of polarized light sensitivity

Since the present results show that polarized light orientation in a number of arthropods depends on a separate sensory input channel, some conclusions may be reached concerning the problem of the optical mechanism of polarized light perception. To begin with, the widespread occurrence of 4 basic steering angles implies that the same or closely similar mechanisms of polarized light perception are present generally in arthropods. This conclusion is consistent with a number of morphological facts at various different levels.

Thus there is remarkable similarity between compound lateral eyes in insects and crustaceans both macroscopically and down to fine details like the primary photochemical pigment involved in light reception and the submicroscopic structure of the rhabdom (Waterman, '60b).

If the generally accepted assumption is made that the ommatidium as a whole is the functional unit for form vision by compound eyes, the only tenable hypothesis for polarized light discrimination as a distinct process is that of Autrum and Stumpf ('50). They postulate that radially differential sensitivity to the *e*-vector of polarized light is shown by the component retinular cells in a single ommatidium. Hence each retinular cell acts as the visual unit for polarized light analysis.

This theory is supported by the electrophysiological results of Lüdtke ('57) and of Burkhardt and Wendler ('60) and has already been used in explaining honeybee responses to the polarized light of the blue sky (von Frisch, '49). Despite negative results reported by two groups attempting to repeat the original electroretinograms (Taylor and Kennedy, '58; de Vries and Kuiper, '58), the Autrum and Stumpf hypothesis remains the only one consistent with the known experimental facts.

In this connection, however, consideration cannot be limited to the compound lateral eye of arthropods. Evidence for polarized light sensitivity in the dorsal eyes of spiders (Papi, '55; Görner, '57) and in simple eyes of water mites (Baylor and Smith, '53; *Arrenurus* in this report) indicates that some simple lens eyes can also perceive this sensory quality.[6]

[6] Less certain evidence is available that the dorsal ocelli of flies (Wellington, '53) as well as the lateral ocelli of certain insect larvae (Wellington, Sullivan and Green, '51) are also sensitive to polarized light.

Although the cellular and organ structure of the various polarized light sensitive eyes may differ markedly, in general they are possession of rhabdoms of closely similar submicroscopic structure (Fernández-Morán, '56, '58, '59; Danneel and Wolitzschel, '57; Goldsmith and Philpott, '57; Miller, '57; Wolken, Capenos and Turano, '57). These facts imply that the presence of this particular kind of photoreceptor element may be correlated with polarized light sensitivity. This general hypothesis is supported by the recent finding that two species of decapod cephalopods (Waterman, Jander and Daumer, unpublished) can perceive the plane of vibration of linearly polarized light. Earlier training experiments on *Octopus* (Moody and Parris, '60) may demonstrate the same point.

Cephalopod eyes, although very different from typical arthropod compound eyes, do nevertheless have structures in the retina which have rhabdom-like micro- and submicro-scopic organization (Grenacher, 1886; Wolken, '58b; Moody and Robertson, '60). Conceivably correlation of eye ultrastructure and basic orientation to polarized light may prove to be an important analytical tool in advancing our understanding of these matters, but the close similarity of the basic responses so far observed in many diverse animals may prevent this.

The facts available suggest that the submicroscopic mechanism depends on oriented photosensitive molecules of dichroic pigment (Stockhammer, '59) as it apparently does in plant cells (Jaffe, '60). However, our knowledge of this subject in arthropods is still far from being at the molecular level. Nevertheless, all eyes known to be capable of effective polarized light analysis contain regularly arranged submicroscopic villi in the probably photosensitive region of the visual cells (Fernández-Morán, '56, '58, '59; Goldsmith and Philpott, '57; Miller, '57; Wolken, Capenos and Turano, '57; Wolken, '58a,b; Stockhammer, '59; Moody and Robertson, '60). These have been postulated to contain oriented layers of visual pigment molecules which might not unreasonably be dichroic. Such results are consistent with the conclusions derived from behavior studies.

D. Light contrast reaction and intensity effect

Comments on this subject will be limited to brief consideration of mechanisms and some discussion of the ecological implications of these relations. The most likely mechanism for the light contrast reaction is that it represents partial or complete inhibition of spontaneous changes in three of the 4 basic steering directions when the vertical illumination is sufficiently more intense than the horizontal.

According to the simplest hypothesis, the intensity effect (p. 143) cannot be mediated by enhancement of the oblique and parallel preferential directions because lowering the overall intensity to 1% has no influence on the spontaneous changes of direction shown by *Daphnia* with the white screened vessel (fig. 4C and D). Rather the reduction in overall light intensity would act by reducing or eliminating the light contrast reaction.

Such an interpretation is supported by two points of comparison between our polarized light data and the phototactic responses of fishes and birds as well as arthropods, described by Verheijen ('58). In both cases the stimulus situation was comparable and the basic orientation was emphasized with increased light contrast. In Verheijen's work, when there was a bright spot in an otherwise dark environment, a strong positive phototaxis[7] was induced and other forms of orientation, such as menotaxis, were inhibited. However, the influence of overall intensity on such light contrast responses was not tested.

An interesting ecological correlation between these two light reactions may be cited: An animal moving towards the sun travels perpendicular to the polarization plane of the overhead blue sky or water so that positively phototactic steering and steering at 90° to the polarization plane would act together under natural conditions. Thus the basic directions induced by

[7] Verheijen, however, claims that an animal orienting itself towards such a light source is disoriented, which seems rather hard to maintain logically whatever its relevance may be in discussing ordinary conditions in a naturally illuminated environment.

the two light contrast reactions are not in conflict but are in fact synergetic.

In relation to normal patterns of natural underwater radiant energy, the present demonstration of strong interactions between basic orientation and both the absolute level of illumination and the differences in its vertical and horizontal intensities shows how important such environmental optical parameters may be for animals' spatial orientation (Waterman, '60e). Radiance distributions, overall intensities and underwater polarization patterns all depend basically on scattering and absorption of daylight by the medium, as well as the depths and wavelengths concerned (Ivanoff, Jerlov and Waterman, '60).

As Verheijen ('58) has emphasized, much of the experimental work on light reactions, particularly of aquatic animals, has been conducted under conditions which differ greatly from those typical of open air or open water. The present data suggest the possibility that quite unexpected patterns of behavior may appear when the illumination of the experimental animals' surroundings is more natural.

E. Integration of polarized light sensory data

The known facts about basic steering directions in polarized light raise some important problems relevant to the animal's choice of a direction of movement. In the present experiments all of the animals showed the individual ability to shift from one preferred orientation direction to another (two in the case of *Mysidium*, 4 for the others).

However, there are interactions among some of the primary directions. Thus only 90° orientation has been observed alone (table 6). When +45° angles appear, so do −45° angles; when 0° angles appear so do 90° angles. As a result, the whole system appears to be made up of two or three partly independent components.

Both external and internal causes may be involved in these changes in steering angle. In the case of internal control of direction there is a tendency to spontaneous (that is, mainly internally caused) changes of basic direction. Although extensive data are lacking, the observed light contrast reaction in *Daphnia* suggests that such spontaneous shifts are favored uniform external conditions. Thus, creasing the differences between the v cal and horizontal luminances augm the spontaneous turning tendency.

Of the various external changes wh may affect the choice of steering a only one is known: namely increasing intensity of vertical relative to horizo light intensity to a point where the l contrast reaction ensues. When this curs, *Daphnia* changes its preferred di tions from any one of 4 to just perp dicular to the plane which course it t maintains. A weaker and less well stu response of the same sort occurred in *sidium* (pp. 145–147) and *Arrenurus* 148), but the experiments on *Hyalella* *Bidessus* were less extensive and yield evidence for such a change.

Although the two visual input chan mentioned above are quite distinct at sensory level and at that of the b steering mechanism, their pathways m converge at higher integrative levels. dence for one kind of higher integra is provided by our data on the light trast reaction (pp. 145, 155–156) wh differences in the angular distributio illumination affect the basic orienta to polarized light.

Evidence for a second high level vergence of intensity pattern and polar light afferent information is available f previous work in a number of cases astronomical orientation. There synch nized daily changes occur in the meno tic steering angles maintained with re ence both to the plane of polarized l and to the direction of highest light in sities (numerous hymenopterans: Frisch, '48, '49; Vowles, '50, '54; Car '51; Jander, '57; Jacobs-Jessen, '59; hemipteran (*Velia*): Birukow and Bu '57; one coleopteran (*Phaleria*): Pa '55; one arachnid (*Arctosa*): Papi, and two littoral crustaceans: one an phipod (*Talitrus*): Pardi and Papi, b; the other an isopod (*Tylos*): Pa '54). An adequate understanding of s integrating mechanisms which control responses to intensity patterns and to larized light awaits further research.

SUMMARY

)irectional responses were tested in 5 cies of aquatic arthropods (three crustans, one insect, one chelicerate) exposed a vertical beam of linearly polarized te light, to horizontal light patterns of rnating light and dark quadrants and combinations of these two.

. Six lines of evidence were obtained ch prove that there are two distinct hanisms for light pattern (image) pertion and polarized light perception. instance, in the dytiscid beetle *Bides* and in *Daphnia* reversing the phototacsign reverses light pattern responses as ected but has no effect on polarized t orientation.

. Under appropriate illumination cons *Daphnia*, the amphipod *Hyalella*, *essus* and the mite *Arrenurus* swim ferentially in the following 4 basic ctions of orientation: parallel, perpenlar and 45° to the left and right of the ctor of the polarized light. Hence we clude that these 4 basic swimming ctions are probably present in most ropods, or at least insects and crustans. Sustained 45° oblique orientation never been observed in light pattern onses.

. Usually our experimental animals nge course frequently and spontausly between the 4 basic directions. if there is strong contrast between h vertical and low horizontal light insities, *Daphnia* tends to orient only perdicular to the *e*-vector of polarized t (*light contrast reaction*). All 4 basic ctions appear if this contrast is less ng or with low overall light intensities *tensity effect*).

ACKNOWLEDGMENTS

Thanks are due to Dr. Nils Jerlov of the anografiska Institutet, Göteborg, for ice on calibrating the light intensity asurements, to Dr. David R. Cook of yne State University for identifying the e species used, and to Mrs. Michael hgarian as well as Dr. William H. cliffe, Jr., Director of the Bermuda Bical Station, for their helpful assistance.

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ffect of Linearly Increasing and Decreasing urrent on the Optic Nerve Discharge of ateral Eye of Horseshoe Crab

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The initial burst and the silent period of e optic nerve discharge in response to latively strong illumination in the dark apted lateral eye of the horseshoe crab as first described by Hartline and Gra- m ('32). The relation between the om- atidial action potential and the frequency the optic nerve discharge was investi- ted by Hartline et al. ('52) with extra- llular recording, and recently was re- amined by means of intracellular micro- ectrode technique (Fuortes, '58; Tomita al., '60; Tanaka and Kikuchi, in prep- ation). From these results, it was shown at the initial burst appeared around the est of the ommatidial action potential, d that the silent period appeared at the irt of the falling phase of the initial polarization.

The effects of direct current flow on the scharge i.e., relation between steady embrane potential and discharge interval ere already reported (MacNichol, '56; iortes, '58; Tomita et al., '60). It seems at the discharge frequency depends not ly upon the membrane potential level t also upon the rate of change of the embrane potential.

In connection with these observations, e effect of linearly changing potential on e discharge interval is a problem to be lved. This report is a study concerned th the accommodation of the optic nerve- dings of the single visual cell, since ere are only a few studies on the accom- odation of single sensory nerve-endings.

METHODS

Materials used were the excised lateral es of the horseshoe crab (*Tachypleus dentatus*) from the Inland Sea of Japan. ie preparation was immersed in the phys- ogical saline solution for the horseshoe crab (Kikuchi and Tanaka, '57). The composition of the solution is as follows: NaCl, 420 mM; $CaCl_2$, 10 mM; KCl, 10 mM; $MgCl_2$, 25 mM; $NaHCO_3$, 2.5 mM.

The experimental arrangements (fig. 1) were essentially the same as before (Tanaka, '60). But in this experiment, the linearly changing current source was used instead of the square pulse generator employed in the previous experiment. The linearly increasing voltage was obtained from an electronic integrator. The principle of the integrator is shown in figure 1 (inside the broken line). The gradient of the linearly increasing voltage was varied by an attenuator connected to the output of the integrator. When a linearly decreasing voltage was applied, the connection of the output of the isolator was reversed. The voltage was applied intracellularly from the isolator through a resistor of 200 megohms and a recording microelectrode. Current flow through the microelectrode, the isolator and the bathing solution due to the resting membrane potential was compensated by a potentiometer inserted in the circuit.

The microelectrodes used were micropipettes filled with 3 M KCl. When the selected electrodes were used, the resistance of the microelectrodes was not significantly changed within the range of currents applied in this experiment. When the current was applied intracellularly, the potential change recorded by application of current was potential drop over the sum of resistance of the cell membrane and the microelectrode. Therefore, the membrane potential was not shown directly on the records. The potential drop over the microelectrode was measured just before the

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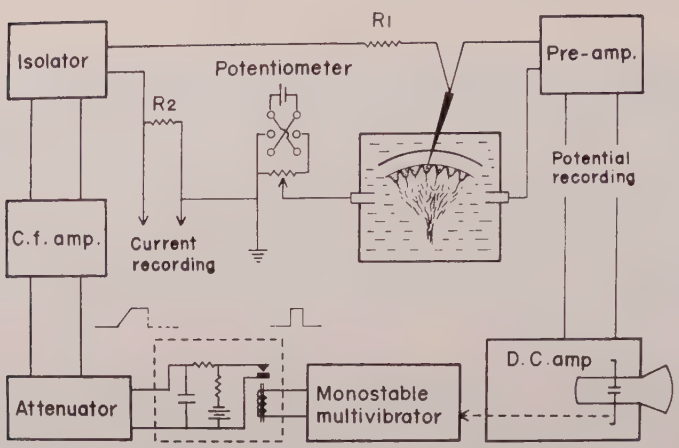


Fig. 1 Experimental arrangement. R_1:200 megohms R_2:1 megohm.

microelectrode insertion to the visual cell, and it was subtracted from the records. The experiments were carried out at 13°C.

RESULTS

The effect of linearly depolarizing and hyperpolarizing current on the spike discharge at various membrane potential levels is illustrated in figure 2. The ordinate indicates the calculated membrane potential levels. The calibration for spike is shown at the bottom of the figure. In record a in the left column, the membrane

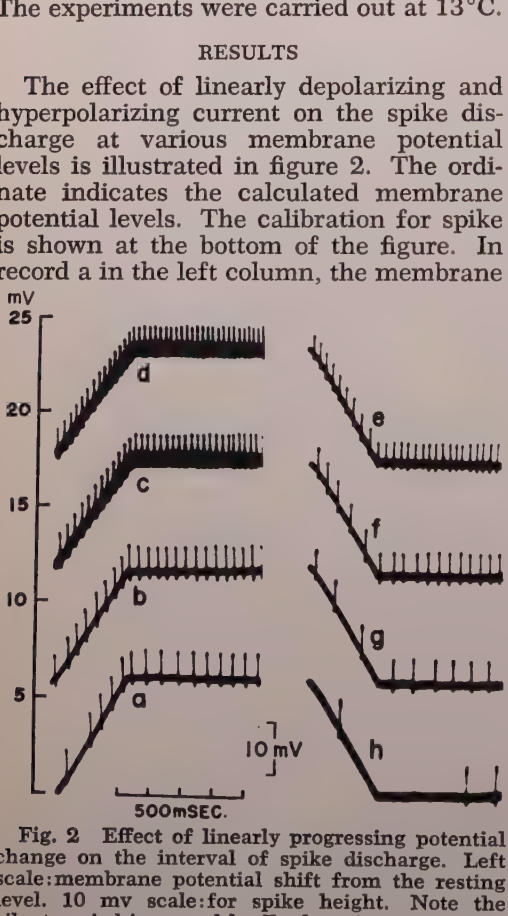
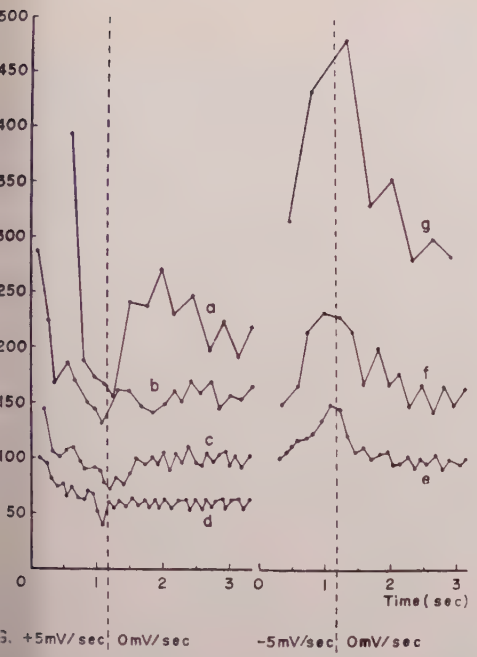


Fig. 2 Effect of linearly progressing potential change on the interval of spike discharge. Left scale:membrane potential shift from the resting level. 10 mv scale:for spike height. Note the silent period in record h. Explanation, see text.

potential was depolarized linearly at t gradient of 5 mv/sec. (depolarization w expressed as positive sign), starting fr the resting membrane potential, th reached to a steady potential level (me brane potential shift of about 6 mv). record b, starting from the steady potent level in record a, the same potential char as in record a was applied. The same tential change was repeated in record and d. The top record in the right colu indicates the potential change which sta from the steady level of record d (ab 23 mv), then was hyperpolarized at gradient of − 5 mv/sec. The same tential changes were applied in sequen until the membrane potential reached t initial resting level.

In figure 3, the discharge interval fr the data shown in figure 2 is plotted agai time. As seen in both figures, even at t same potential level, the discharge int vals during the course of the linearly p gressive depolarization and hyperpolari tion and also at the early part of t steady potential level following a linea potential change are remarkably differe The discharge intervals during linear polarization and at the steady potent level of records in the left column smaller than those of records in the ri column at the same potential level. Th differences are greater at more hyperpol ized potential levels.

At a steady potential level after a me brane potential change, the discharge terval increases or decreases gradually a



ig. 3 Discharge interval in relation to time, ted from the data of figure 2. Broken line, l point of the linearly progressing potential. radient of potential change.

ches an approximately constant value. l at more depolarized potential level, time required to reach a constant dis- rge interval is smaller. Even at the ie potential level, the time following ar depolarization is smaller than that owing hyperpolarization.

t was shown in this experiment that the charge interval at each steady potential l depends on the membrane potential l as reported before (Tomita et al., '60). fluctuation of the discharge interval steady potential level is larger at more olarized potential levels.

DISCUSSION

is well known that the receptors are pheral transducers which respond to a cific stimulus by generating afferent ve impulses. If the adequate stimulus pplied and continued, the receptor re- nds to the maintained stimulus with a t of nerve impulses whose duration es considerably among the different ptors. In the visual organ, adaptation s place slowly and the spike discharge inues for as long as the visual organ is illuminated, although the frequency of discharge tends to diminish.

The frequency of spike discharge rises from low values to a high value as the intensity of illumination is increased. However, factors concerned in the determination of the discharge frequency are the time course of the generator potential and the type of the afferent nerve. In this experiment, the response of the optic nerve-ending to the potential change of various gradients and at different potential levels can be compared with those obtained from other nerve fibers in response to linearly increasing currents.

According to the study on the relationship between the discharge interval and membrane potential level previously reported by Tomita et al. ('60; see also fig. 4 in Tanaka, '60), the effect of potential change on the spike discharge increases as the membrane is more hyperpolarized. The relation between the change in the excitability of the optic nerve and the discharge interval is already discussed (Tanaka, '60).

The discharge interval depends not only upon the membrane potential but also upon the time course of the potential change, i.e., even at the same potential level, the discharge interval during the course of the linear depolarization is different from that during hyperpolarization. From this relation and the results in the previous report, it is easily understood that the excitability of the optic nerve-ending during depolarization is different from that during hyperpolarization at the same potential level.

Differences between discharge intervals during depolarization and hyperpolarization are larger at more hyperpolarized potential level.

As quantitatively shown in the previous paper, the change in the excitability required to cause a certain change in the discharge interval of the optic nerve reduces as the memebrane is more hyperpolarized (Tanaka, '60). Hence, on the contrary, difference in excitability in related to time course of potential change is rather smaller at more hyperpolarized potential level than that at depolarized potential level.

The discharge interval at a steady potential level fluctuates to a wider extent

when the membrane is more hyperpolarized. From the relation mentioned above, it seems that the fluctuation of the excitability of the optic nerve-ending at hyperpolarized potential level is not larger than that at depolarized level.

The silent period and the burst of the spike discharge similar to those observed during the initial maximal depolarization of the ommatidial action potential could be demonstrated by intracellular application of extrinsic currents. It is deduced from this experiment that the gradient of hyperpolarization or depolarization and the membrane potential level are the limiting factors for the appearance of the silent period or the burst of the spike discharge caused by the extrinsic current. If these factors are considered on the discharge superimposed on the ommatidial action potential, a relatively large initial depolarization—which is obtained when the resting membrane potential is large and the visual organ is stimulated by illumination of relatively high intensity and after long time in darkness—is the important factor for the appearance of the initial burst and the silent period of the optic nerve impulses by illumination.

SUMMARY

The effect of linearly progressing depolarization and hyperpolarization on the optic nerve-ending of the lateral eye of the horseshoe crab (*Tachypleus tridentatus*) was studied by intracellular microelectrode.

Even at the same potential levels, the discharge intervals during linear depolarization were smaller than those during linear hyperpolarization. These differences in the discharge interval which depends upon the gradient of potential change were greater at more hyperpolarized potential levels.

At the beginning of the steady potential level following the linear depolarization, the frequency rate of discharges showed the maximum, then tended to decline and finally reached an approximately constant value which is a function of the membr potential. On the other hand, it was m mal at the beginning of the steady tential level following the linear hy polarization, increased gradually and tained an almost constant value. The t required to reach a constant discharge terval increased as the membrane more hyperpolarized.

The discharge interval of the optic ne ending fluctuated even at the steady tential levels. This fluctuation was m remarkable at more hyperpolarized tential level.

The relation between excitability discharge interval of the optic nerve-end and the limiting factor for the appeara of the initial burst and the silent perio the optic nerve discharge observed du illumination were discussed.

ACKNOWLEDGMENT

The authors wish to express our tha to Dr. R. Kikuchi, Department of Phy ogy, Tokoyo Women's Medical College his valuable discussions in preparing paper.

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daptive Changes in Trimetaphosphatase of louse Intestine[1]

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Trimetaphosphatase catalyzes the hydolysis of trimetaphosphate to inorganic ipolyphosphate. The enzyme was found a wide variety of vertebrates (Berg and easer, '53; Berg, '55, '60; Berg and Gorn, '60), including man. In all of these was abundant in the epithelia of digesve organs and scarce elsewhere, except r a high concentration in the proximal nvoluted tubule of the kidney. This disibution of trimetaphosphatase in the verbrate body is practically diagnostic of ssues derived from endoderm.

Such an anatomical distribution can be ausibly explained in two ways: (1) *imetaphosphatase is a functionless vesge of a lost metabolic pathway.* In the own metabolic pathways trimetaphosate can originate only from the breakwn of linear polyphosphates of long ain length (Thilo, '55). Metazoan mebolism does not utilize either such linear lymers (above $n = 4$) or trimetaphosate. More primitive organisms, on the her hand, utilize high polymer polyphosates as energy storage compounds (Ebel al., '58; Wilkinson, '59) and there trietaphosphate may be a normal metabite. Trimetaphosphatase may, therefore, a working enzyme in protista, but only vestigial one in the metazoan endoderm. 2) *Trimetaphosphatase is a working enme in metazoa, and it is required for at ast one kind of active transport between e external and the internal environment.* his theory accounts for the anatomical stribution of the enzyme in epithelia enged in active transport, but requires stulating a new metabolic pathway. The sociation of trimetaphosphatase with ace transport is shown even in the two gans where trimetaphosphatase is not the endoderm. One is the kidney, where trimetaphosphatase is always in the mesodermal epithelium of the proximal convoluted tubule (Berg, '60). The second is the small intestine of the rat where in some strains trimetaphosphatase is completely absent from the epithelium, but where in all these cases it is abnormally high in the mesodermal lamina propria mucosae (Berg and Gordon, '60), as if a function abandoned by one tissue layer was taken over by the one next to it (Herrmann, '60).

One way of discriminating between a vestigial marker and a functional enzyme is to find out whether the level of enzyme activity changes adaptively with the function which it is supposed to serve (Knox, et al., '56), or whether it remains constant as a morphological marker of a differentiated cell. In this study, we assayed trimetaphosphatase in the intestines of fed, starved, and refed mice, and found that trimetaphosphatase activity changed adaptively with function.

MATERIAL AND METHODS

1. *Treatment of animals.* Young, adult, female mice of the Swiss Webster albino strain were used. Controls were fed Purina Laboratory Chow. Starved animals were offered either water alone or water and a meal of cellulose, for 60–70 hours. Starvation beyond 70 hours was not well tolerated by the mice during the fall and winter months. Refed animals ate a humid mash of three parts of glucose to 7 parts of cellulose (Tuba and

[1] This investigation was supported by Research Grant A-1089(C-3) from the Institute of Arthritis and Metabolic Diseases of the U. S. Public Health Service and aided by the University of Rochester Atomic Energy Project, Rochester, N. Y., under contract with the Atomic Energy Commission.

Dickie, '54) and were sacrificed 4 hours after they started eating.

The effect of starvation and refeeding on trimetaphosphatase was studied in 18 fed, 15 starved, and three refed animals. Six of these fed and 8 of these starved animals were used for the alkaline phosphatase assay; 8 more fed mice were assayed for alkaline phosphatase only.

2. *Biochemical assay of trimetaphosphatase.* The mouse was decapitated, opened, and examined. Animals with signs of disease were discarded, and so were "unfed" animals with a bolus in the stomach or intestine, and "fed" animals with no bolus in the digestive tract. Approximately one quarter of the small intestine of sound animals was mobilized starting at the pyloric sphincter. This portion was flushed in situ with warm isotonic saline, removed, cleaned of fat and mesenteries, and flushed again. Length and wet weight were taken after a mild blotting, and the specimen was suspended in an aluminum foil sling in a bath of liquid nitrogen. The remainder of the small intestine was then removed, measured, and discarded. The parts of the intestine used for assay averaged 28% of the total length (S.D. = ± 4%).

The frozen specimen was coarsely crushed in a mortar pre-chilled in dry ice, and transferred before thawing to a Tenbroeck homogenizer (12 cm^3). All subsequent operations were done on ice with pre-chilled reagents. The specimen was homogenized in distilled water with no more than 10 strokes, and allowed to settle. The supernatant was then decanted into a small beaker of coarse anion-cation exchange resin (4 meq of Dowex 1×8, OH form, 20–50 mesh and 2½ meq of Dowex 50×10, H form, 20–50 mesh), stirred, and decanted into a graduate cylinder. Water was added to the residue in the homogenizer and the entire procedure was repeated until there was no residue left after decanting. The mortar, homogenizer and resin were then rinsed with 10 cc of 0.01% Triton X-100 and with a final rinse of distilled water. All supernatants and rinses were pooled in the cylinder, 10 cm^3 of 1×10^{-2} M Na_2H_2 EDTA (ethylenediamine tetraacetate) were added, and the volume was made up to 50 cm^3 with distilled water (Berg and G don, '60). Part of this stock solution diluted 1:1 without changing the conc tration of Triton and EDTA (the dilut fluid was 8×10^{-3}% Triton and 2×1 M EDTA), and trimetaphosphatase ac ity was measured at each of the two c centrations of the homogenate.

Where enzyme activity was high, stock solution was diluted twice (1:1 1:3) and the homogenate measured both concentrations.

Dry weight was determined in 4- samples of the homogenate dried 16 hc at 130–140°C and then dessicated *in va* over calcium chloride for two ho Weight was corrected for the Triton EDTA remaining after drying.

Incubation was carried out for 45 m utes at 38°C in stoppered 25-cm^3 Er meyer flasks. The incubating mixt consisted of 2 cm^3 of homogenate, 1 of 0.2 M acetate buffer, pH 4.5, and 1 of substrate solution (82.25 mg% of dium trimetaphosphate, corresponding 250 µg P/cm^3). Phosphate assay controls were done as described by B and Gordon ('60). Measurements w normalized to 25% of length of the sm intestine.

3. *Biochemical assay of alkaline ph phatase.* Alkaline phosphatase was tes in two preparations. (a) The stand preparation consisted of the stock hom enate suitably diluted with distilled wa (up to 10 × dilution). (b) The ED free homogenate was prepared with wa replacing EDTA and was similarly dilut The incubation mixture was 1 cm^3 hom enate, 1 cm^3 fresh substrate (Na_2 p-ni phenyl phosphate, Sigma, 150 µg P/cm and 1 cm^3 of pH 10.3 buffer (0.1 M cine NaCl) (Ohmori, '37). Sufficient m nesium acetate was added to the buffe give a final concentration in the incu tion mixture of 1.3×10^{-3} to 1.4×1 M/l of magnesium salt, after correct for binding by EDTA. The substrate buffer were brought to 38°C in a stoppe 25 cm^3 Erlenmeyer flask, the homogen was added, and the mixture was incuba for 30 minutes. The reaction was stop by adding 2 cm^3 of 0.1 N NaOH and c ing. The cold mixture was centrifug the supernatant made up to 10 cm^3

sured in a Lumetron colorimeter with arrand interference filter transmitting 92 mμ ± 10 mμ. The readings given enzyme-free control tubes were sub- ted from the experimental readings to a measure of enzyme activity (Bessey, ry, and Brock, '46). The method was itive to less than 2 μg of orthophos- te P.

Histology and histochemistry. Par- sections of the intestine were stained trimetaphosphatase (Berg, '60). Epi- ial counts were made in 6 μ cross- ions of the pyloric end of the small stine stained with hematoxylin and n. For cell counts a section was pro- ed on a photomicrographic screen itz optics, with Periplan ocular and IVb photo head). The outline of all the magnified 136 × was traced on onion paper, and the length of the outline asured with a chartometer (Keuffel and er model 214, 100-cm dial). Four in each section outline were marked measured individually from base of ot to tip, and their epithelial nuclei e counted on the screen under 136 × 612 × magnification. The total num- of epithelial cells in the section was puted by multiplying this count by the o of total inner perimeter to perimeter measured villi. Two different sections each intestine were measured and nted.

RESULTS

1. *Properties of enzymes*

he pH optimum for trimetaphosphat- in mouse duodenum was pH 4.5 (fig. Specific activity of trimetaphosphatase dry weight) was independent of the fold dilution of enzyme. Overnight age of frozen homogenate at −25°C owed by thawing caused a loss of tri- aphosphatase activity, and more en- e was lost when the homogenate was ted before storing (88% of activity ained in a 100 cm^3 dilution, 75% in 0 cm^3 dilution, 68% in a 400 cm^3 dilu-). Activity was also lost if the excised stine was allowed to autolyze before freezing (some 50% loss after a 45- ute delay at room temperature).

lkaline phosphatase was activated by nesium salts with an optimum con- ration of 1.3 to 1.4 × 10^{-3} M. At the

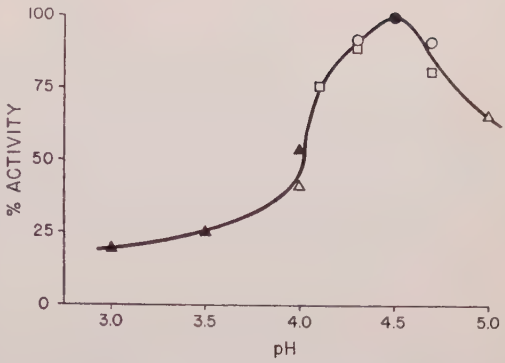

Fig. 1 pH curve of intestinal trimetaphosphatase. Enzyme activities are shown as % of the activity of the same homogenate at pH 4.5. Each point is a duplicate determination, and each symbol (△, ●, □) represents one animal . .. Filled-in symbols show the use of alanine buffer in place of acetate.

standard substrate concentration (p-nitrophenyl phosphate equivalent to 150 μg P per tube) the pH optimum was 10.3. The rate of hydrolysis was not significantly influenced by the loss of substrate in the course of the incubation (for substrate concentrations ranging from 200 μg P/tube to 100 μg P/tube, the apparent loss of activity was less than 3% for each decrement of 10 μg P of substrate per tube, and the enzyme concentrations were adjusted to keep the total hydrolysis below 10 μg P/tube). Cation exchangers inactivated the enzyme, and activity was not completely restored when the homogenate was supplemented with magnesium. At their optimum Mg^{++} concentrations, the homogenates prepared with EDTA had only some 12% of the activity of similar homogenates prepared without EDTA.

2. *Adaptive changes of enzyme activity*

Biochemical changes. The dimensions of small intestines of fed, starved and refed mice are shown in figure 2 and the wet weight and some 40% of the dry changes are summarized in table 1. Starvation caused the loss of some 25% of the wet weight and some 40% of the dry weight in the anterior 25% of small intestine, without altering the length.

The decrease of trimetaphosphatase activity on starvation was even greater, with less than half of the enzyme remaining

after starvation. Refeeding for 4 hours did not alter the weights, but it did bring about a partial return of enzyme activity (fig. 2 and table 1).

The activity of alkaline phosphatase also decreased with starvation (table 2). The overall decrease in total activity was significant on the 5% level. This decrease was less than the concomitant decreases of wet and dry weight, so that when zyme activity was computed on the b of weight, the loss of enzyme caused starvation was masked by the loss weight.

Histochemical and histological chan Sections of duodenum of fed anim reached the full stain for trimetaphosp tase in 1½ hours or less (fig. 3a). W

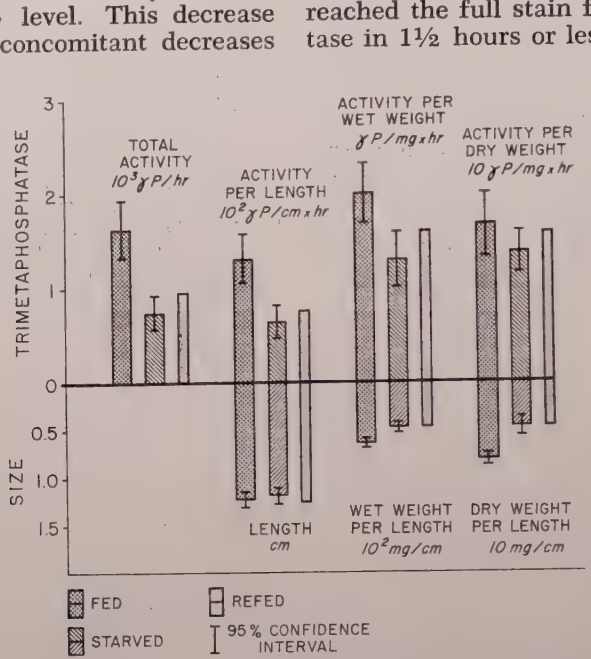

Fig. 2 Effect of standard diet, starvation, and refeeding of glucose on the anterior quarter of small intestine of mice. Mean values for trimetaphosphatase and 95% confidence intervals of the means are shown above the abscissa. Mean values for the dimensions of the intestine in the same animals are shown below the abscissa, together with 95% confidence intervals of means.

TABLE 1

Changes in trimetaphosphatase in the small intestine of the mouse after starvation and refeeding

	Loss on starving	p	Gain on feeding	p
	%		%	
Size				
Total length	− 3	n.s.	+ 7	n.s.
Wet weight per cm	−25	0.001	0	n.s.
Dry weight per cm	−42	0.001	+ 2	n.s.
Tritmetaphosphatase				
Total activity	−54	0.001	+29	0.02
Activity per wet weight	−35	0.001	+23	n.s.
Activity per dry weight	−18	n.s.	+14	n.s.

The percentage change after starvation is the difference between mean fed value and mean starved value converted to % mean fed value. The percentage change after refeeding is the difference between mean starved value and mean refed value, converted to per cent of mean starved value. The level of significance of the difference (p) is shown as n.s (not significant) where $p \geqslant 0.05$.

duodenum came from a starved mouse, staining took twice as long. Figure shows early (incomplete) staining in a ved mouse after 1½ hours of incuba-. When starved mice were refed glu- mash, the speed of staining returned ards normal three to 4 hours after a al, but not earlier.

The number of cells in the intestinal epithelium of a starved mouse is compared with that of a fed mouse in table 3. The starved animal had less dry weight, wet weight and enzyme, but did not have any fewer epithelial cells than the fed animal. The difference of trimetaphosphatase activity between the two animals was great-

TABLE 2

Effect of starvation on alkaline phosphatase in the small intestine

Enzyme preparation	Mouse		Rat (from literature)
	Homogenate with EDTA	Homogenate without EDTA	Extract
Ratio of total activities	0.77 ± 0.15	0.79 ± 0.23	0.6 to 0.8
Ratio of activities per wet weight	1.41 ± 0.15	1.01 ± 0.40	1.1
Ratio of activities per dry weight	1.58 ± 0.28	1.23 ± 0.51	—

The mouse data are for the anterior 25% of small intestine, and show ratios of mean alkaline phosphatase activity after starvation to that after feeding, together with ± S.D. of the ratio. The ratios in rats are computed from Madsen and Tuba ('52) and Tuba and Robinson ('53) for the anterior 10 cm of small intestine after 6 to 12 days of starvation.

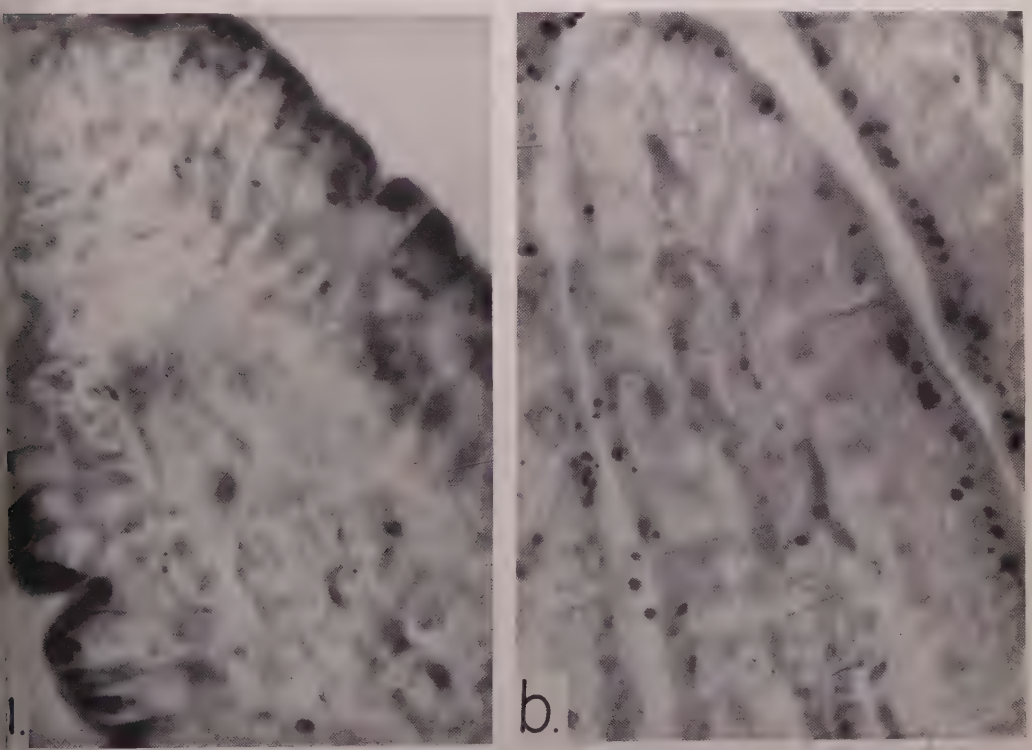

Fig. 3 Effect of starvation on intracellular trimetaphosphatase in duodenal epithelium. Both sections were incubated one and one-half hours. (a) Villus of fed mouse. Full stain. (b) Villus of starved mouse. Partial stain.

TABLE 3

Comparison of anterior quarter of small intestine in a fed and a starved mouse

Measurement	Units	Fed mouse	Starved mouse
Number of cells of columnar epithelium	$\frac{10^3 \text{ cells}}{6\ \mu \text{ length}} \pm$ range	8.1 ± 0.2	12.5 ± 1.0
Inner perimeter	mm ± range	37.9 ± 0.8	34.7 ± 0.2
Length	cm	11.5	13.8
Wet weight/length	mg/cm	67.9	35.7
Dry weight/length	mg/cm	8.9	5.9
Trimetaphosphatase			
Total activity	$10^3\ \gamma$ P/hr.	1.22	0.69
Activity/length	γ P/cm × hr.	106.0	50.0
Activity/dry wt.	γ P/mg × hr.	11.9	8.5
Activity/cell	$10^{-6}\ \gamma$ P/cell × hr.	7.9	2.4

est when it was computed per enzyme-bearing cell.

DISCUSSION

The adaptive response of intestinal alkaline phosphatase to feeding was first found in the rat (Bellini and Cera, '40) and has been extensively studied in that species since then. The rat duodenum was, therefore, the logical choice for an inquiry into adaptive responses of trimetaphosphatase. However, the initial tests of trimetaphosphatase in the rat duodenum showed that the enzyme had an aberrant distribution, as compared to the distribution in two species of fishes, two of amphibians, one bird embryo and two other species of mammals. Typically, trimetaphosphatase is localized in the columnar epithelium of the duodenum, but in two of the rat strains tested (Wistar and Agouti-Fisher) it was altogether absent from the epithelium, and in two other strains (Sprague-Dawley and a gutter rat) it was shared by the epithelium and the underlying stroma cells of the lamina propria.

Mice had a standard localization of both trimetaphosphatase and alkaline phosphatase in the intestinal epithelium, and a change of alkaline phosphatase staining with diet has been reported (Deane and Dempsey, '45) but the quantitative response of mouse alkaline phosphatase to starvation and feeding was unknown. Fortunately, the quantitative response of alkaline phosphatase to prolonged starvation turned out to be the same in mice as it was in rats (Madsen and Tuba, '52; T and Robinson, '53; table 3). In both cies some alkaline phosphatase acti was lost on fasting, but the loss wa greater than the overall loss of mass. the absence of a direct quantitative h chemical assay (Casselman, '59) o differential cell counts such as the presented here, this drop in enzyme a ity could just as well be ascribed to specific cellular attrition (Miller, '48 to an adaptive response. The publi biochemical evidence that alkaline p phatase in rat duodenum changes a tively with digestion was derived from effects of feeding on the enzyme ra than from effects of starvation.

The response of trimetaphosphata starvation was greater than that of line phosphatase and greater than change of weight, so that the duode lost trimetaphosphatase selectively, f than it lost overall bulk. This ada loss of activity was shown in two wa be a response of individual cells of digestive epithelium rather than a s tive destruction of cells in the o (Gadzhiyeva, '57). The counts of ep lial cells showed that the decrease i metaphosphatase from a fed to a sta animal was not due to the decreas number of enzyme-bearing cells, whil histochemical stain gave a direct, if s quantitative, demonstration of the crease of enzyme activity within each

The increase in trimetaphospha activity after an ad libitum meal of cose was similar to, although lower

e increase in alkaline phosphatase ac-
ity shown after such a meal in rats
uba and Dickie, '54). The response pre-
ded any gain in weight, and the localiza-
n of the increase of trimetaphosphatase
thin the epithelial cells was confirmed
histochemical staining.

The three hour delay in the response to
eding shown in histochemical stains of
metaphosphatase was comparable to a
lay of more than three and less than
hours reported for the increase of alka-
e phosphatase in similar material
eane and Dempsey, '45). The mice in
e latter experiment were on a complex
nedule (24 hours of starvation, three
urs of refeeding, followed by prolonged
starvation), which yielded the surpris-
g result that the intensity of alkaline
osphatase staining continued to increase
the first three days of restarvation.

We can conclude, that the columnar epi-
elial cell of the intestine changes its
ntent of trimetaphosphatase in response
changes of function. After a glucose
al, the columnar epithelial cell syn-
sizes trimetaphosphatase in the apical
oplasm. The same cell has been re-
rted to change volume and to form
nular inclusions in the apical cyto-
sm (Ritter, '57), at least some of which
e Hale's stain positive and periodic acid-
niff positive (Müller, '56). At the same
ne, in all likelihood, the amount of alka-
e phosphatase increases in the cell mem-
ane above the apical cytoplasm, and in
e Golgi zone directly below. In the
sence of a theory of transport of metab-
tes across epithelium that would ac-
unt for any of these events (de Duve,
), we must include trimetaphosphatase
nong the possible components of the
ive transport mechanism.

SUMMARY

Trimetaphosphatase activity (pH 4.5)
mouse duodenum decreased 54% on
rvation, while wet weight decreased
% and dry weight 42%. Alkaline phos-
atase activity in the same homogenates
creased approximately 20%. Trimeta-
osphatase was demonstrated histochem-
lly only in columnar epithelium. The
mber of these enzyme-bearing cells was
lowered by starvation, while trimeta-
phosphatase activity per cell decreased and
the histochemical staining reaction of
epithelial cells was weakened.

Feeding of glucose to starved mice
raised trimetaphosphatase activity approx-
imately 30% in 4 hours. The pronounced
response to fasting and prompt response
to feeding are evidence for the presence
of an adaptive link between trimetaphos-
phatase activity in the duodenum and the
functioning of the digestive tract.

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ome Aspects of Fungal Bioluminescence[1]

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The emission of light from fungal spe-
es has been intermittently studied for a
nsiderable period of time. In the fungi
minescence is confined, in the main, to
e Basidiomycetes with one species, Xy-
ria in the class Ascomycetes possibly be-
g luminous (Harvey, '52). In some in-
ances both the mycelium and fruiting
dy is luminous, i.e., *Omphalia flavida;*
others only the mycelium is luminous,
., *Armillaria mellea.*

Information concerning the mechanism
fungal bioluminescence is on the whole
actically nonexistent. That oxygen is
cessary for light emission for these or-
nisms was established by Robert Boyle
the 17th century, and this has been re-
nfirmed by several investigators (Kawa-
ura, '15; Buller, '24; Nobecourt, '26; Har-
y, '26; Bothe, '28). Thus, in this respect,
e fungi are similar to the majority of the
minous organisms. This requirement in-
cating that respiratory energy is involved
the emission of light has been found
r both the luminous bacteria and the
efly systems (Harvey, '52).

The emission spectra for the luminous
lds have been studied (Coblentz and
ghes, '26) and, as reported by Harvey
2), by van der Burg. Since the latter
blication was not readily available, this
operty has been reinvestigated.

Harvey ('52), Buller ('24), Kawamura
5), Ewart ('07) have all reported their
successful attempts to obtain a cell-free
ht emitting system for the luminous
ngi. Recently this was accomplished
irth and McElroy, '59). Former failures
n now be explained on the basis that one
the requirements for light emission is
duced pyridine nucleotide. The effect is
nspecific, in that the reduced form of
her diphosphopyridine or triphosphopyri-
ne nucleotide is functional at the present
ge of purification of the luminous sys-
n. In addition to the above requirement
e classical heat labile enzyme, luciferase,
and a hot water component, presumably luciferin, and oxygen are required for light emission. During the course of isolating the cell-free system, several experiments of a physiological nature were conducted and these are now reported.

MATERIALS AND METHODS

Cultures

The original culture of the luminous mold, *Armillaria mellea,* was kindly supplied by Dr. P. Snider, then of Harvard Biological Laboratories. All cultures were grown on slants consisting of 3.6% (w/v) mycophil agar (Baltimore Biological Laboratory) and 0.5% (w/v) yeast extract (Difco). This medium was adjusted to pH 5.5 with 0.1 N HCl, autoclaved and used as such. The organisms were grown at 18°C.

The culture of the luminous bacterium *Photobacterium fischeri* was orginally supplied by Dr. W. D. McElroy of The Johns Hopkins University. It was grown on a modification of the medium suggested by McElroy and Farghaly ('48) which consisted of trace elements, 0.05 ml; NaCl, 30 gm; $Na_2HPO_4 \cdot 7H_2O$, 5.3 gm; KH_2PO_4, 2.1 gm; $(NH_4)_2HPO_4$, 0.5 gm; $MgSO_4$, 0.1 gm; glycerol, 3.0 ml; nutrient agar, 24 gm; H_2O, 1000 ml. The pH was adjusted to 7.3. Trace elements were identical to those recommended by McElroy and Farghaly ('48). The bacteria were also grown at 18°C.

Light emission was quantitatively measured with an instrument designed by Dr. E. F. MacNichol of The Johns Hopkins University. This instrument utilized a 931 A photomultiplier tube and a Sanborn Model 151 single channel recorder was employed to record the light intensities.

Emission spectra were measured on an Aminco-Bowman spectrophotofluorometer.

[1] This work was supported in part by research grants from the National Science Foundation (G-6430) and from the National Institutes of Health (RG-5989).

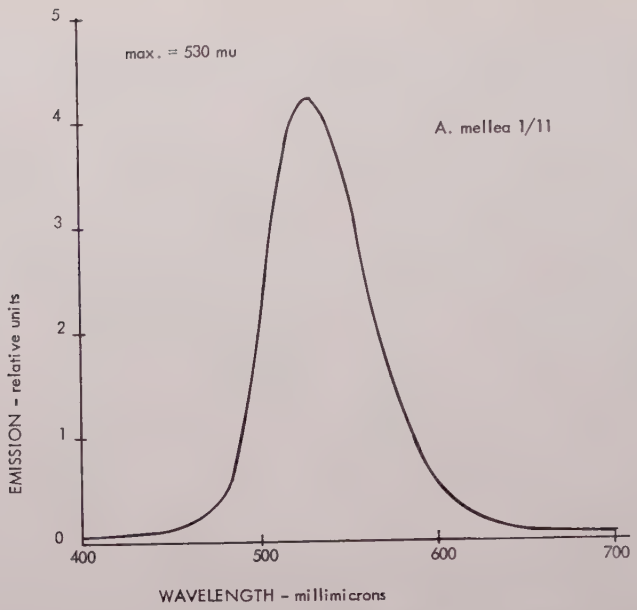

Fig. 1 The emission spectrum of the luminous mold *Armillaria mellea.*

RESULTS AND DISCUSSION

1. *Emission spectrum.* The emission spectrum of a 15-day-old culture of *A. mellea* is presented in figure 1. The apparent maximum emission was recorded at approximately 530 mμ; no instrument corrections have been made. Some impression of the intensity of light emission may be gained from figure 2. A plus X film was exposed to a culture of the same age for 5 minutes. The camera lens was three feet removed from the surface of the petri plate for this photograph. It is of interest to note that the young outer reaches of the mycelium are considerably more luminous than the older central portions of this structure.

The recorded emission maximum of 530 mμ agrees with the value of 528 mμ published by van der Burg for *A. mellea, Mycena polygramma* and *Omphalia flavida.* An emission maximum of 520 mμ has been recorded for *Polyporus honedai, Mycena bambusa,* and *Pleurotus lenaillustris* and *Agaricus medens* as indicated by Harvey ('52). The emission maximum for the luminous molds is on the blue side of that of the firefly which is at approximately 565 mμ and to the red of the luminous bacteria which has an emission around 480 mμ.

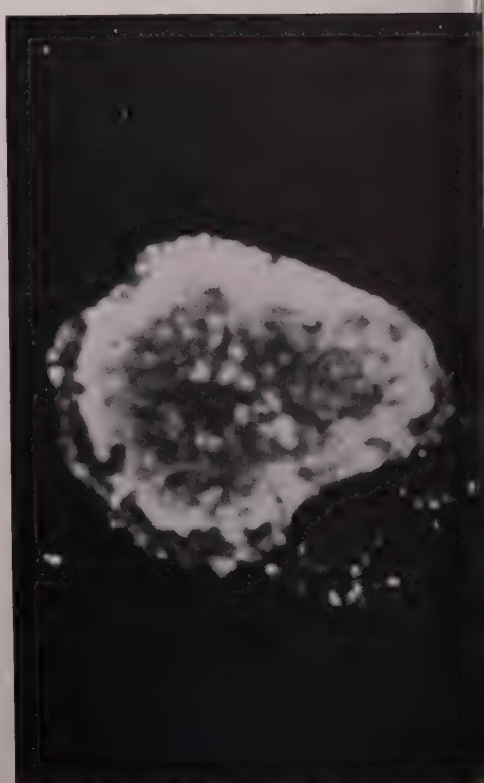

Fig. 2 "Self photograph" of *A. mellea.* Emission from *A. mellea* was used as a light source

The 530 mμ light produced by the lumi-ous molds corresponds to about 55 kilo-alories per einstein. The chemical nature f the molecule undergoing excitation and ubsequent light emission is unknown at he present time. Spectral and fluorescent haracterization of a crude hot water ex-ract from which one would expect to find he luciferin, has been studied. This prep-ration was made by extracting an ace-one powder of *A. mellea* with 0.05 M otassium phosphate buffer, pH 7.0, con-aining 10^{-3} M ethylene diamine tetraace-ic acid. Several absorption and fluores-ent peaks occur in such an extract; the nost prominent having an apparent ab-orption maximum at 420 mμ and a fluo-escent maximum at 480 mμ. Whether his compound represents mold luciferin is nknown at the present time. The fact hat its fluorescent maximum does not cor-espond with the chemiluminescence max-mum need not be disturbing. This case nay be similar to the firefly system where hemiluminescence is 15 mμ to the red of he fluorescence of both luciferin and luci-eryl-adenylate (Rhodes and McElroy, '58).

2. *Effect of temperature.* The effect of emperature on fungal bioluminescence as studied by determining the light in-ensity at room temperature (23°C). The lant was then equilibrated for 15 minutes n a constant temperature water bath and he emission intensity at this temperature etermined. A different slant was used for ach determination. An Arrhenius plot f the log of the per cent of the original ght intensity against the reciprocal of the bsolute temperature has been made and s presented in figure 3.

The "temperature optimum" for the uminous molds is at 26°C under the condi-ions of measurement, this value corre-ponding to the *in vitro* optimum for the refly system of 25°C (McElroy and Streh-er, '49). The temperature optimum for he luminous bacteria depends upon the pecies but all have optima between 15 to 0°C. The slope of the curve in figure 3 nay be used to calculate the energy of ctivation and this value is 17,500 calories. imilar calculation for the *in vitro* firefly ioluminescent reaction has been reported s 18,500 calories (McElroy and Strehler, 8). Brown, Johnson, and Marsland ('42) eported a value of 17,000 calories for the

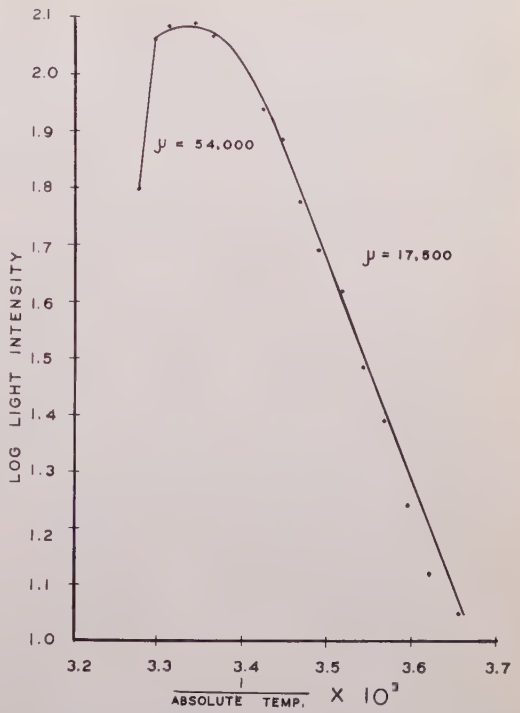

Fig. 3 Arrhenius plot of light emission from the luminous mold *A. mellea* at different temperatures.

heat of activation for *Photobacterium phosphoreum*. It is interesting to note that the energy of activation for the mold is intermediate between that of *Photobacterium* on one hand and the firefly on the other, just as is the emission maximum. At temperatures greater than 28°C, the heat of activation is 54,000 calories and this value is identical with the given for the above mentioned bacterial system for temperatures above optional.

3. *Effect of ultraviolet light.* During the course of purifying mold luciferase the results suggested that the enzyme was readily inhibited. The initial purification stages of both the bacterial and firefly systems have presented similar difficulties (McElroy, '58). In the case of the bacterial system exposure of bacterial luciferase to ultraviolet light has two effects, namely destruction of an inhibitor—possibily riboflavin—and the activation of a component necessary for light emission (McElroy, et al., '54).

The response of the luminous mold *A. mellea* to 3660 A light (Black Ray Lamp)

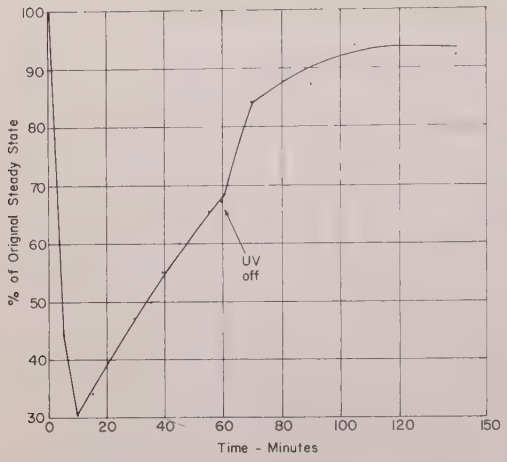

Fig. 4 Effect of ultraviolet light on bioluminescence from the luminous mold *A. mellea*.

is presented in figure 4. In this figure the original steady state value has been assigned as 100%. Adequate controls were run to assure that the effects measured were due to irradiation and not thermal. During the exposure to ultraviolet light there is first a rapid decline followed by a gradual increase in light emission. The increase becomes more pronounced when the light source is removed. The new steady state in the presence of ultraviolet light is less than that achieved when it is removed. This new steady state, both in the presence and absence of the ultraviolet source may or may not be greater than the original steady state. The level is determined by the particular culture under consideration and the intensity of the incident ultraviolet light. That these bioluminescent responses are a function of light intensity may be seen from table 1. In this case the slants were irradiated for 15 minutes with var ing intensities of ultraviolet light and th reduction in the steady state of bioluminescence determined. The increases in ligh emission were determined 45 minutes aft the ultraviolet source had been remove *In vitro* experiments have convicingly der onstrated that the luminescent activity the hot water extract is readily destroye by ultraviolet light (Airth, '60). Simil exposure of an *in vitro* enzyme preparatio does not have this effect; indeed there ma be a slight stimulation of enzymatic acti ity when exposed to ultraviolet light.

Interpretation of these data are open several possibilities, one of which wou be: there are two photo-labile compoun present in the *in vivo* system. The o compound is essential for bioluminescen and this accounts for the reduction in lig emission. The other photo-labile co pound is an inhibitor of bioluminescen which when destroyed would account f the increase in light emission. The tim required to reach a new steady state eith during or after irradiation would be function of such factors as the rate of sy thesis versus the rate of destruction of t photo-labile factors. Countering this pro ess which tends to reduce bioluminescence would be the rate of destruction the presumed inhibitor which would te to increase light emission. Another, a equally valid interpretation of the data, that instead of postulating an inhibitor compound(s) is photo-chemically produc which is required for light emission. present there is no evidence for selecti one of these possibilities in preference the other.

TABLE 1

The response of A. mellea to different intensities of 3660 A irradiation

The original steady state value has been assigned as 100% and all other values compared to this. Light emission after 15 minutes of ultraviolet irradiation at different intensities was determined. Bioluminescence was again determined 45 minutes after the ultraviolet source had been removed.

3660 A intensity relative units	% Reduction in original steady state during UV exposure	% Increase over original steady state after UV exposure
16	39.5	67.0
7.1	23.0	75.0
1.8	14.0	3.0
1.0	0.0	0.0
0	0.0	0.0

4. *Response to oxygen.* The response the luminous fungus *A. mellea* to oxygen s been studied and these responses com- red to the luminous bacteria *Photobacter- m fischeri.* In these experiments, slants the culture were exposed to the desired s mixture of oxygen and nitrogen. The s was kept flowing at a rate of 39 ml per cond. Under these conditions the gas as replaced in approximately 0.5 seconds. hen the slants were exposed to anaerobic nditions, tank nitrogen was used with no tempt being made to remove the trace nounts of oxygen (approximately 0.02%) at occurs in this source. The nitrogen as also flowing at a rate of 39 ml per cond. Preliminary experiments had indi- ted that a difference in the flow rate of e oxygen mixture (oxygen + nitrogen) d the nitrogen caused emission results om the organisms when switching from e gas to the other not attributable to the mposition of the gas itself. The molds ere exposed to three minutes of 20% ygen before use and the bacteria for 5 nutes at the same gas concentration. is precaution was found necessary in ew of the great reduction in light emis- n while 20% oxygen was being passed er the slant. This reduction in light ission amounted to approximately 80% the original light intensity in the case of e fungi and about 50% in the case of the cteria.

The reasons for this reduction in light emission on flowing gas over the bacterial or mycelial surface is not clear. The possibility that the cultures were being desiccated due to the high rate of gas flow was readily eliminated by bubbling it through columns of water before passing over the culture. Also the possibility that this reduction in bioluminescence is due to lowering of the temperature because of evaporation seems very unlikely. The results of figure 3 can be used to calculate that there would have to be a 15°C reduction in temperature to account for the decrease in bioluminescence that was observed in the case of the molds. The system was also checked to determine if it was developing a pressure within the culture tube and this was found to amount to only 4×10^{-3} atmospheres greater than normal. It may be that this reduction in bioluminescence is ascribable to input pressure which amounted to 30 pounds per square inch at the exit of the flow meters used. Even this possibility does not seem too reasonable since Brown, Johnson, and Marsland ('42) found that pressure in the region of 6000 pounds per square inch was required to appreciably reduce *P. fischeri* bioluminescence at 25°C. All gas experiments were done at 23°C.

The "flash" of the luminous mold is presented in figure 5. The mold was exposed to 30 seconds of nitrogen just previ-

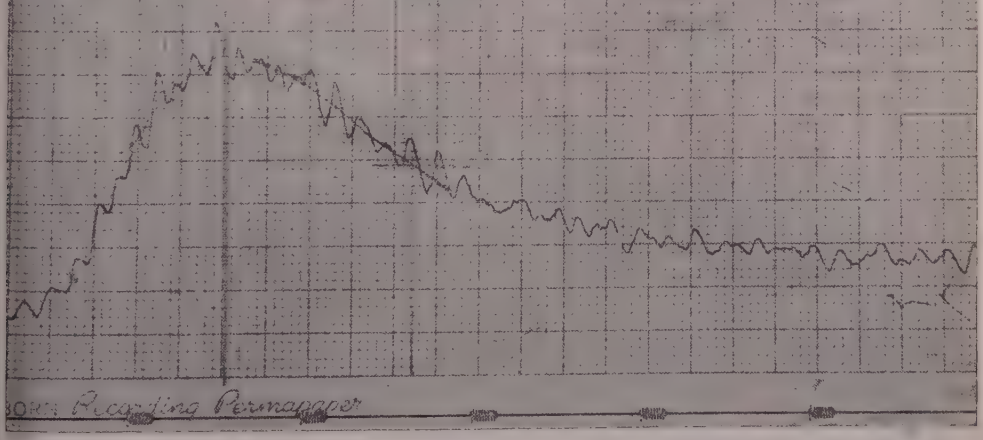

Fig. 5 Recording of flash of light from luminous mold *A. mellea* after 30-second treatment with 100% nitrogen and then exposure to 20% oxygen. All gases were flowing at a rate of 39 ml/second. See text for explanation.

ous to the admission of 20% oxygen and 80% nitrogen. In this case, an adaptor was placed in front of the photomultiplier tube which reduced its light-accepting area to 2 mm² and hence the signal noise is very large. As the rate constants determined either with or without the adaptor were about the same the adaptor was not utilized further. The intensity of the flash is a function of the length of nitrogen exposure as can be seen in table 2. The slants were exposed to 20% oxygen after the nitrogen and the maximum intensity of the flash recorded. The data for the luminous bacteria have also been added and it can be seen that the mold response is about twice that of the bacteria on a comparative basis.

TABLE 2

The effect of different periods of anaerobiosis on flash intensity

Light intensity in 20% oxygen flowing at a rate of 39 ml/sec. was determined. Slants were then exposed to 100% nitrogen flowing at the same rate for varying time periods and then to flowing 20% oxygen. The maximum light intensity recorded during the oxygen treatment is compared to the initial steady state light intensity.

N_2 treatment	Per cent of original steady state	
	A. mellea	*P. fischeri*
seconds		
15	580	236
30	600	267
60	690	262
120	658	281
180	716	375
300	482	355

Another factor that affects the characteristics of the light emission after anaerobiosis is the composition of the gas. Table 3 presents the half lives for b the increase and the decay of the flash tained after 30 seconds of nitrogen. half life has not been presented for build up of the 20% oxygen mixture. experimental arrangement was of suc nature that at lower oxygen concentrat the gas mixing was inadequate to meas initial rates accurately. The values p ented are the averages of 6 successive terminations done on the same slant v 30 seconds of nitrogen alternating with seconds of oxygen. A fresh slant was u for different oxygen concentrations. T could be measured accurately to 0.05 onds. These data suggest that the in reactions in the bioluminescent flash limited by comparatively high oxygen centrations under the conditions utili The rate limiting reaction during the d portion of the flash does not appear t affected by the oxygen concentration. other feature of the half lives in the cas both organisms is that this paramete independent of the intensity of the f as is indicated by table 4.

Harvey ('35), Johnson, van Schouw berg and van der Burg ('39), Cha Harvey, Johnson and Millikan ('40) Hastings ('52) have all proposed sche to account for the reactions of biolumi cence. The series of reactions prop by Chance et al. ('40) consisted of following:

$$LH_2 + A \rightarrow LH_2 \cdot A$$
$$LH_2 \cdot A + \tfrac{1}{2} O_2 \rightarrow LH_2 \cdot A \cdot O$$
$$LH_2 \cdot A \cdot O \rightarrow A^* + L + HOH$$
$$A^* \rightarrow A + \text{light}$$

TABLE 3

Effect of different oxygen concentrations on half-lives of emission flash after nitrogen treatment

Slants were exposed to nitrogen flowing at a rate of 39 ml/sec. for 30 seconds and then to differing concentrations of oxygen flowing at the same rate. The half-lives of the increasing and decaying portion of the bioluminescent flash have been recorded.

Oxygen concentration	*A. mellea*		*P. fischeri*	
	Half-life of increase	Half-life of decay	Half-life of increase	Half-life of decay
%	*seconds*	*seconds*	*seconds*	*seconds*
100	0.12±0.05	1.40±0.10	0.41±0.04	2.93±0.62
80	0.15±0.06	1.29±0.14	0.46±0.09	2.76±0.48
60	0.29±0.08	1.17±0.07	0.50±0.05	2.68±0.44
40	0.64±0.12	1.12±0.06	0.66±0.08	2.52±0.28
20	—	1.34±0.12	—	2.20±0.25

TABLE 4

Independence of half-life of emission on intensity of emission

Slants were exposed to 20% oxygen flowing at a rate of 39 ml/sec. for equilibration. They were then exposed to 100% nitrogen for 30 seconds and then 100% oxygen. All gases were flowing at a rate of 39 ml/sec. The half-lives of the increasing and decay portion of the emission flash are compared to the maximum intensity of emission.

A. mellea			*P. fischeri*		
Flash height relative units	Half-life of increase	Half-life of decay	Flash height relative units	Half-life of increase	Half-life of decay
	seconds	*seconds*		*seconds*	*seconds*
160.0	0.10	1.22	600	0.43	3.02
120.0	0.11	1.42	420	0.45	4.00
90.0	0.08	1.50	360	0.47	2.75
70.0	0.10	1.35	270	0.43	2.80
56.0	0.10	1.40	240	0.43	2.75
51.2	0.20	1.52	180	0.40	3.27

In this proposal luciferin, LH_2, reacts ith luciferase, A, to form an enzyme subrate complex. This complex then reacts ith oxygen which in turn forms an exted complex A*. With the emission of a ght quantum free luciferase is again rermed. Under anaerobic conditions reacn (1) proceeds and $LH_2 \cdot A$ accumulates; en when oxygen is introduced (2), reacns (3) and (4) proceed. Johnson et al. 39) observed in their studies with the minous bacteria that under extended riods of anaerobiosis, i.e., 10 minutes, e initial flash was reduced in intensity d this was followed by what they termed "secondary rise" in the light intensity. is later effect was a comparatively slow crease in bioluminescence followed by a w decline. To account for this effect ey suggested that luciferase was reversiy broken down by proteolytic activity of e bacterial cell. Hastings ('52) observed a nilar secondary rise in the case of the minous mold *Panus stipticus* with the ect being much more striking than in e case of bacteria. Other experiments this same publication were consistent th the postulate that luciferase had two tive sites specific for the substrates ciferin and oxygen. If one of the subates was absorbed on the wrong site it ted as a competitive inhibitor.

Schoepfle ('40) carried out a detailed alysis of the kinetics of the flash of *P. cheri* at different temperatures. On the sis of the fact that light emission is indendent of oxygen concentration between and 380 mm of mercury for *Cypridina*, hoepfle concluded that reaction (2), above, was for all practical purposes instantaneous. Reaction (3) results in an increase in the concentration of A* and reaction (4) in a decrease. Reactions (3) and (4) are two consecutive first order reactions and the light intensity, I, will correspond to the rate of breakdown of A*. Solving as two differential first order equations Schoepfle obtained the expression for light intensity at any time:

$$I = \frac{k_3 k_4}{k_4 - k_3} \cdot (A \cdot LH_2 \cdot O)_0 \cdot (e^{-k3t} - e^{-k4t})$$

where k_3 and k_4 are the velocity constants for reactions (3) and (4) respectively. The velocity constants were calculated from the relationship:

$$\frac{Y}{2X} \pm \frac{1}{2}\sqrt{\frac{Y^2}{X^2} - 4\left(\frac{Y^2}{X^2} - \frac{Z}{X}\right)} = a, b$$

where

$$k_3 = -\frac{1}{t} \ln a$$

$$k_4 = -\frac{1}{t} \ln b.$$

In this case $a = e^{-k3t}$, $b = e^{-k4t}$ and X, Y, Z, are light intensities at times t, 2t and 3t. This approach for the solution of k_3 and k_4 has two major disadvantages. The values for X, Y and Z must be of such numerical value that $\frac{Y^2}{X^2} > 4\left(\frac{Y^2}{X^2} - \frac{Z}{X}\right)$ such will not be the case if $X > Y > Z$. Also the values of a and b would have to be less than one or the velocity constants will have negative values. These two requirements for the solution k_3 and k_4 place considerable restrictions on the solution of their values.

Since the above analysis is for two consecutive first order reactions where:

$$I = \frac{k_3\ (LK_2 \cdot A \cdot O)_0}{k_4 - k_3} \cdot (e^{-k3t} - e^{-k4t}) \qquad (5)$$

the following may offer a simpler solution for the evaluation of k_3 and k_4. Using the expansion:

$$e^{-x} = 1 - x + \frac{x^2}{2!} - \frac{x^3}{3!} \cdots$$

(5) will become:

$$I = \frac{k_3\ (LH_2 \cdot A \cdot O)_0}{k_4 - k_3} \times$$

$$\left[(1 - k_3t + \frac{k_3t^2}{2!} \ldots.) - (1 - k_4t + \frac{k_4t^2}{2!} \ldots.)\right]$$

which will simplify and approximate to

$$I = \frac{(LH_2 \cdot A \cdot O)_0\ k_3t\ (k_4 - k_3)}{k_4 - k_3}$$
$$= (LH_2 \cdot A \cdot O)_0\ k_3t.$$

On rearranging

$$k_3 = \frac{I}{(LH_2 \cdot A \cdot O)_0 \cdot t}. \qquad (6)$$

Thus the initial light intensity may be utilized to evaluate k_3.
Then let

$$B = \frac{A^*}{(LH_2 \cdot A \cdot O)_0},\ K = \frac{k_4}{k_3},\ \text{and}\ T = k_3t\ .$$

When

$$\frac{dB}{dT} = 0$$

then

$$k_3t_{max} = T_{max} = \frac{\ln K}{K - 1}.$$

Since t_{max} is determined experimentally and k_3 has been evaluated, then a plot of K against T_{max} can be used to evaluate K. When the value for K is known the relationship:

$$k_4 = k_3K \qquad (7)$$

may be used to solve for k_4.

The velocity constants k_3 and k_4 have been calculated using the method proposed above and these values are presented table 5. Since, under the experiment co ditions used, oxygen becomes limiting comparatively high concentrations (s table 3) these constants have been calc lated for cultures that were exposed 100% oxygen. The values presented a satisfactory for comparative purposes or and are probably much smaller than t actual velocity constants. An appreciati of the greater rates possible, at least the case of bacteria, may be gained fr the fact that both Chance et al. ('40) a Schoepfle ('40) measured the time to rea maximum emission at approximately 0. seconds which is 5 times as rapid as t values reported here. However, the ra constants for reaction (3) is about times as rapid in *A. mellea* as in the ba teria. This suggests that the reacti velocities for the mold and Cypridina, measured by Chance et al. ('40) are a proximately similar in magnitude.

The analysis carried out by Schoep ('40) and Johnson et al. ('39) postulat that $LH_2 \cdot A$ would accumulate during a aerobiosis. This possibility was verified measuring the total light emitted duri the experiment rather than measuring lig intensity. Table 6 presents the results total light measurements for slants of *mellea* and *P. fisheri* exposed to nitrog for varying periods of time and then 20% oxygen. A fresh slant was used f each nitrogen exposure. In each case t cultures were equilibrated against 20 oxygen flowing at a rate of 39 ml per se ond for 7 minutes. Total light emissi was recorded for the next three minut and then the slant was exposed to nitrog after which it was exposed to 20% oxyge The light emission during the three mi

TABLE 5

Comparison of velocity constants k_3 and k_4 in A. mellea and P. fischeri
Experimental procedure as in table 4. See text for description.

	A. mellea	*P. fischeri*
Total light, relative units	5334	5148
t_{max}, seconds	0.54	1.55
Intensity at t = 0.25 sec.	5.7	0.8
k_3	4.3	0.06
T_{max}	1.07	0.014
K	0.90	70
k_4	3.8	4.3

TABLE 6

The effect of varying periods of anaerobiosis on total light emission from A. mellea and P. fischeri

Slants were equilibrated in 20% oxygen and then exposed to varying periods of nitrogen. Nitrogen treatment was followed by exposure to 20% oxygen. All gases were flowing at a rate of 39 ml/sec. See text for description of calculations.

Time of nitrogen exposure	*A. mellea*		*P. fischeri*	
	Predicted light units	Actual light units	Predicted light units	Actual light units
seconds				
15	54.27	55.72	115.6	94.32
30	42.57	51.70	170.6	161.6
60	40.68	54.52	112.5	114.3
120	46.7	48.3	93.2	120.2
180	203.4	209.9	179.8	105.7
300	67.2	57.4	149.4	90.5

s previous to nitrogen treatment was ed to predict light emission during the sequent 10-minute period. This latter minutes included the nitrogen treatnt. The predicted values are compared h the actual experimental values obned during the latter 10-minute period. ntrol experiments indicated that this thod of calculating light emission was curate to no more than 5% in the molds d 10% in the bacteria. The data do subntiate the concept that LH_2 will acnulate during short periods of anaerosis. However, with longer nitrogen osures the data suggests that luciferin roken down by non-light emitting reions.

Whether the reactions proposed initially Harvey ('35) and Johnson et al. ('39) modified by subsequent authors nance et al., '40; Hastings, '52) actually resent the *in vivo* reactions of bioluminence is open to conjecture. The kinedata agree with the proposed schemes. ently Rhodes and McElroy ('58) while estigating the reactions of the firefly em found that oxidized luciferin, oxyferin, was very tightly bound to the ferase to form luciferase-oxyluciferyldenylate complex with a dissociation conit of 5×10^{-10}. The rate constant for dissociation of this complex into free ferase and oxyluciferyl-adenylate was $\times 10^{-3}$ sec^{-1}. The stability of the ene complex accounted for the very rapid luct inhibition of the bioluminescent tions in the *in vitro* firefly system. Pro- inhibition has not been considered to gnificant degree in any of the kinetic ysis carried out to date. It is quite conceivable that the dissociation of an enzyme-product complex could be of significance in both the bacterial and mold systems also. The data suggesting that luciferin accumulates during anaerobiosis could be as readily explained by postulating that the concentration of the active intermediate $LH_2 \cdot A$ is controlled not by luciferin accumulation exclusively but also by the rate of dissociation of an enzyme-product complex. Indeed it is possible to interpret the "secondary rise" observed by Johnson et al. ('39) and Hastings ('52) on the basis of an equilibrium between the reactants:

$$A + LH_2 \rightleftharpoons A \cdot LH_2 \quad (8)$$

$$A \cdot LH_2 + O_2 \rightleftharpoons A^* \cdot L + H_2O \quad (9)$$

$$A^* \cdot L \rightarrow A \cdot L + \text{light} \quad (10)$$

$$A \cdot L \rightleftharpoons A + L \quad (11)$$

where L represents oxyluciferin. If the intermediate $A \cdot LH_2$ accumulates during anaerobiosis the extent of this accumulation will depend upon the equilibrium constants of reactions (8) and (11). On the introduction of oxygen there will be a "flash" of light; its intensity depending upon the concentration of $A^* \cdot L$. The reaction kinetics for this scheme predicts the possible oscillatory behavior of light emission as observed by Johnson et al. ('39) for bacteria and Hastings ('52) for the luminous mold. If the dissociation constant of reaction 11 is very small as it is in the case of the firefly system, then luciferase could conceivably be rate limiting and a decrease in light emission would be observed. Dissociation of the oxyluciferyl-luciferase complex would release free enzyme which in turn could react with the excess luciferin that had accumulated during the

anaerobic treatment thus accounting for the secondary rise.

SUMMARY

Various characteristics of the luminous mold *Armillaria mellea* have been investigated and in some instances compared with similar characteristics of the luminous bacterium *Photobacterium fischeri*. Emission from the luminous mold is at approximately 530 mμ corresponding to about 55 kilocalories per einstein. The energy of activation for the mold was found to be equal to 17,500 calories with the "temperature optimum" at 26°C. Exposure of the fungi to 3660 A irradiation causes an immediate reduction in bioluminescence with a subsequent increase. The possible significance of these findings has been considered. Also the response of both the mold and bacteria to oxygen after short periods of anaerobiosis was studied and considered in the light of previous experimental results.

ACKNOWLEDGMENT

The authors wish to express their deep appreciation to Dr. R. K. Clayton of Oak Ridge National Laboratories for his assistance in the kinetic analysis of reactions 8–11.

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THIS NUMBER COMPLETES VOLUME 56

JOURNAL OF

CELLULAR AND COMPARATIVE PHYSIOLOGY

VOL. 56 DECEMBER 1960 No. 3

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PRESS OF
THE WISTAR INSTITUTE
OF ANATOMY AND BIOLOGY
PHILADELPHIA

Printed in the United States of America